Genetic Diversity and Structure of Galápagos Mockingbird Populations and Species (*Mimus* spp.)

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CONTENT

GENERAL INTRODUCTION

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Genetics of small island populations

Islands have long fascinated scientists, and island populations have been the focus of many important evolutionary studies both in the past (Berry, 2009, Darwin, 1839) and present (Grant, 1998). Island populations provide ideal systems to study fundamental topics of population genetics which aim at disentangling the different evolutionary forces that lead to the observed pattern of genetic variation within and between populations and species. Because of their isolation and restricted geographic range, but also because extinction and re-colonization events are generally more frequent on islands, island populations often display lower levels of genetic diversity than mainland populations (Frankham, 1997). Decreased variation in island populations and increased differentiation among them may result from founder events that occurred at the time of colonization (Clegg et al., 2002) and the long-term effects of genetic drift (Mundy et al., 1997).

Genetic drift, i.e. the random changes in allele frequencies and the accompanying fixation and loss of particular allelic variants over time, is commonly the most powerful evolutionary force acting in small populations (Crow and Kimura, 1970). However, as complete avoidance of mating between relatives is impossible in populations of finite size, genetic drift cannot be observed without inbreeding (Kristensen and Sorensen, 2005). Hence, increased genetic load due to drift (Wright, 1931, Lynch et al., 1995), together with the expression of inbreeding depression (Keller and Waller, 2002) are potential genetic causes for reduced fitness in small populations. Fitness reduction following loss of genetic diversity and inbreeding has been observed in a number of wild populations (e.g. Hogg et al., 2006, Fredrickson et al., 2007, Lacy, 1997) and such negative effects have been shown to be especially pronounced under stressful environmental conditions (e.g. Keller et al., 2002, Ross-Gillespie et al., 2007, Bijlsma et al., 2000, Coltman et al., 1999). As a result of the loss of adaptive genetic variation, smaller populations are furthermore less capable of adapting to novel environmental challenges and lose evolutionary potential. Synergistic interactions with demographic or

environmental stochastic events may individually or combined lead to a further decrease in population size, which ultimately may result in extinction (Frankel and Soulé, 1981). This is why small populations, such as island populations, are of conservation concern.

An additional explanation for increased extinction risk in small populations is that reduced genetic diversity makes them more vulnerable to infectious diseases (McCallum and Dobson, 1995, Hudson et al., 2002). For example, heterozygosity has been suggested to correlate with host resistance to parasite infections (Puurtinen et al., 2004, O'Brien and Evermann, 1988, Watkins et al., 1991). Thus, inbreeding in animals may increase their susceptibility to disease. Small host populations on isolated islands are particularly sensitive to foreign pathogens (McCallum and Dobson, 1995) and disease has been implicated as a major factor leading to population declines and extinctions on islands (e.g. Van Riper *et al*., 2002).

Loss of genetic diversity caused by genetic drift and inbreeding can be counteracted by gene flow between populations, i.e. the migration and subsequent reproduction of individuals, which has a homogenizing effect among populations. Gene flow can add new or replace lost alleles (Slatkin, 1985), increase genetic diversity and evolvability (Houle, 1992) and hence decrease extinction risk. However, gene flow can also constrain evolution and prevent local adaptation (Postma and van Noordwijk, 2005). If genetic drift and gene flow are in equilibrium, then the rate of change is slowed down relative to what would be expected under drift alone, and genetic diversity and structure are maintained. Therefore, from an evolutionary as well as conservation perspective, it is interesting to determine the role of genetic drift and gene flow on levels of genetic diversity and structure.

Conservation programs aim to minimize inbreeding and loss of genetic diversity (Caballero and Toro, 2000) and secure the survival of small populations by increasing the total number of individuals and/or by (re-)establishing gene flow among isolated populations (e.g. Hedrick, 1995, Pimm et al., 2006). Population size, however, may not always be a good predictor of genetic diversity because it does not necessarily reflect effective population size (N_e), i.e. the amount of the gene pool passed on to the next generation (Franklin, 1980) which hence also serves as an estimate for the rate of loss in

genetic diversity (Crow and Kimura, 1970). Molecular information can be used to estimate N_e and determine the magnitude of drift or gene flow that has occurred in any particular population by examining the level of molecular divergence and diversity (Chakraborty and Nei, 1977, Wright, 1978). Furthermore, the analysis of historic samples can provide an insight into the past and allows the molecular analysis on a temporal scale to assess past and present levels of genetic diversity and N_e (Wandeler et al., 2007).

Integrating knowledge of effective population size and temporal gene flow and drift is a vital part for understanding patterns of population genetic variation to a) differentiate between recent or ancestral effects and b) determine how much genetic diversity is needed for the long-term persistence of populations. Such information can then be used in conservation management to predict problems associated with inbreeding depression (Groombridge *et al.*, 2009) in endangered species or restored populations.

This thesis aims to contribute to our understanding of the effects of genetic drift and gene flow on the genetic diversity of different-sized populations on a temporal and spatial scale. Furthermore, the relationship between genetic diversity and estimates of immunocompetence as a measure of disease susceptibility is investigated to assess whether inbreeding has an effect on a fitness-related trait. This study investigates these effects on two scales, a) on an archipelago-wide scale by studying many populations of all four species of mockingbirds endemic to the Galápagos Islands, and b) specifically for an endangered species, the Floreana mockingbird, for which some of the findings may have conservation management implications.

The mockingbirds of Galápagos

The Galápagos Islands lie about 1000 km off the west coast of Ecuador, and since 1959 approximately 97% of their terrestrial habitat is protected by the Galápagos National Park. It is the only remaining conserved tropical archipelago in the world with over 95% of its biodiversity intact (CDF and WWF, 2002). However, the islands' ecosystem has been impacted by the human presence and introduction of non-native

species, especially on the inhabited islands. It was probably a combination of habitat alterations following human colonization and introduced predators such as black rats and cats (CDF, 2008) that led to the disappearance of the Floreana mockingbird, *Mimus trifasciatus*, on Floreana Island at around 1880 (Curry, 1986). Today the Floreana mockingbird is classified as "Critically Endangered" by the IUCN Red List of Threatened Species, with only approx. 400 individuals left, restricted to two satellite islands off Floreana (Fig. 1): Champion with 20-50 individuals (Grant et al., 2000) and Gardner-by-Floreana with an estimated 300-500 individuals (PEA Hoeck and LF Keller; unpublished data; also see *Mockingbird Census Methodology*). To protect this species from extinction, a reintroduction plan has recently been developed (CDF, 2008).

Based on phenotypic differences, besides *Mimus trifasciatus,* there are three other species of mockingbirds recognized in the Galápagos (Harris, 1974), all endemic to the islands and living allopatrically (Fig. 1): *Mimus macdonaldi* on Española and its satellite Gardner-by-Española, *Mimus melanotis* on San Cristóbal, and *Mimus parvulus* which inhabits all other major islands of the archipelago except Pinzón, Baltra and North Seymour.

The Galápagos mockingbirds likely differentiated within the archipelago in the last five million years (Arbogast et al., 2006), hence relatively recently, but in contrast to other species such as the Darwin's finches, no sympatry has evolved so far. The mockingbirds are hypothesized to be relatively weak fliers as they have rarely been seen flying over water or appear on islands where they don't breed (PR Grant and RL Curry, personal communication). Their allopatric distribution on islands of different sizes, most of which still provide an intact and little impacted habitat, makes the mockingbirds an ideal system to investigate the relationship between genetic diversity and population size, and determine the effects of drift and gene flow on the diversification between populations.

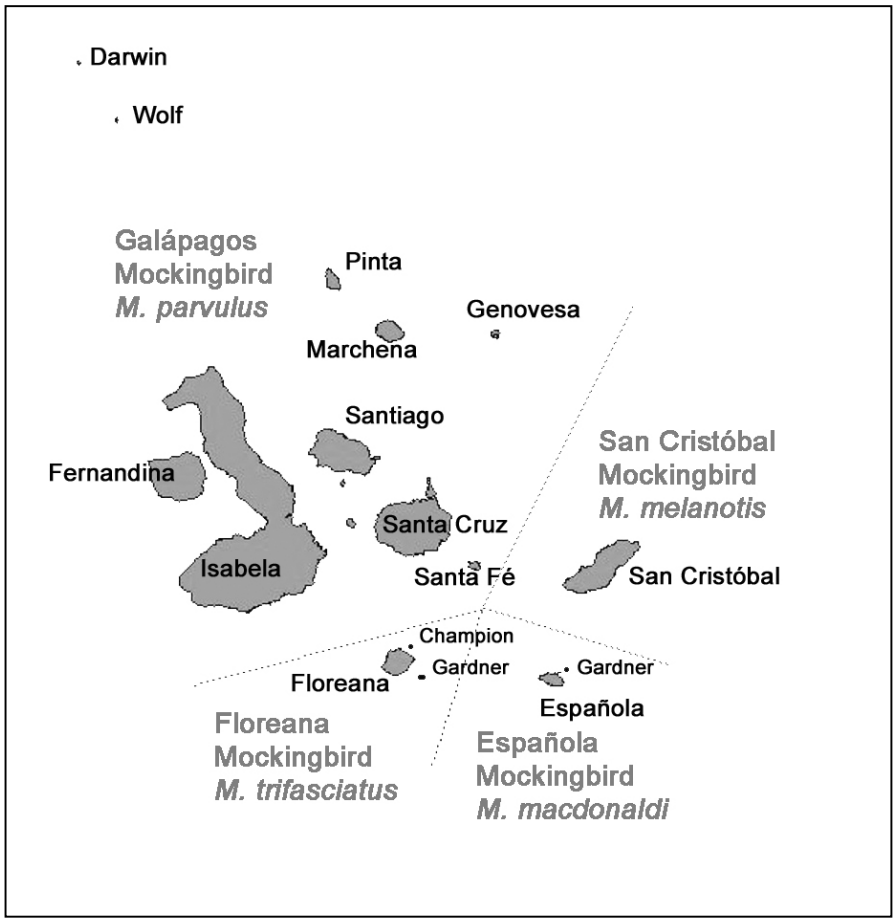

Figure 1: Distribution of the four mockingbird species in the Galápagos.

Measuring genetic diversity and inbreeding

There are two ways of measuring inbreeding in natural populations: pedigree analysis and molecular genetic analysis. As pedigree analysis requires parentage information over several generations (Keller and Waller, 2002), which is rarely available for natural populations such as the Galápagos mockingbirds, here I used neutral genetic markers (microsatellites) to estimate inbreeding.

Multilocus heterozygosity is the most useful parameter for estimating genetic diversity as it can be compared across species (Toro and Caballero, 2005). Heterozygosity from neutral genetic markers, such as microsatellites, has been shown to

often correlate with nucleotide diversity in non-coding regions on a population level, even when a correlation is absent at the individual level (see below). It is therefore believed that neutral genetic markers provide a reasonable estimate for inbreeding at the population level (Väli et al., 2008).

Measuring inbreeding at the individual level with genetic markers has been debated because the comparison of inbreeding coefficients from pedigree and genetic data in domestic and wild animals showed that individual heterozygosity from neutral genetic markers may only correlate weakly with inbreeding coefficients from pedigrees (e.g. Balloux et al., 2004, Slate et al., 2004, DeWoody and DeWoody, 2005, Aparicio et al., 2007). Therefore, and because there were no heterozygosity-heterozygosity correlations for the microsatellite loci at the individual level (Chapter 4), I decided against analyses using multilocus heterozygosity as an estimate for individual inbreeding (Coltman and Slate, 2003).

Measuring immunocompetence

An individual's immunocompetence is based on the three major defense mechanisms of the immune system (i.e. innate, humoral and cell-mediated immunity) and is defined as its ability to prevent or control infection by pathogens and parasites (Norris and Evans, 2000). Therefore, immunocompetence is used as a measure of the birds' susceptibility to disease. As higher levels of one component of the immune system need not imply greater overall resistance (Adamo, 2004, Matson et al., 2006) and there may be trade-offs between different defense mechanisms (Norris and Evans, 2000), simultaneous measurement of multiple immune parameters should be performed when ever possible (Keil et al., 2001, Adamo, 2004). I tested the innate immunity in populations of all four Galápagos mockingbird species by counting different types of white blood cells (leucocytes), a method widely used in avian studies to measure stress, and the innate humoral immune response by assessing natural antibody and complement enzyme activity. Innate defenses are constitutive and induced rapidly and therefore most important against first exposures to pathogens and quickly growing infections. A link

between genetic diversity and innate immune response, such as levels of natural antibodies (Parmentier et al., 2004), seems likely, like it has been shown for other immunologically important proteins (Miller and Lambert, 2004). Also, natural antibodies have been suggested to provide a link between the innate and acquired humoral immunity (Lammers et al., 2004, Ochsenbein and Zinkernagel, 2000), and hence may possibly also predict the strength of the adaptive immune response (Kohler et al., 2003).

Thesis outline

Chapter 1 is a technical paper in which I present the development of Galápagos mockingbird specific microsatellite primers which provided the basis for all further genetic studies described in this thesis. The isolation of microsatellite loci in *Mimus parvulus* and the design of primer pairs that bind equally well onto sequences in all four mockingbird species (i.e. show similar peak intensity) was crucial to avoid potential artificial differences in genetic diversity estimates, i.e. ascertainment bias that can result from the selection for polymorphism during marker development (Brandstrom and Ellegren, 2008). In cross-species comparisons, such ascertainment bias can lead to artifactual differences because the loci will be more polymorphic in the species in which the microsatellite loci were developed (e.g. Ellegren et al., 1995). With the design of the 20 microsatellite loci described in this chapter, ascertainment bias among the four mockingbird species in Galápagos was deliberately avoided by including all loci that were polymorphic in at least one of the species. Furthermore, in light of the work with historic specimens, primers were designed specifically to obtain short microsatellite products to improve amplification success with degraded DNA.

In **Chapter 2** I examine the genetic diversity and differentiation of 19 different mockingbird populations on a temporal and spatial scale, covering the range of all four species and using the microsatellite markers described in Chapter 1. I used contemporary DNA collected in the field from 2006-2008 as well as historic DNA dating back to the early 1900 for almost all extant mockingbird populations. The chapter describes the

genetic relationship between the four mockingbird species and the distribution of genetic diversity within and among mockingbird populations to estimate the effects of genetic drift versus gene flow between populations. I determined how island size and isolation affect the genetic structure of mockingbird populations and how well population size can be inferred from genetic diversity. To assess the role of population size in maintaining genetic diversity over time, I estimated variance effective population size by determining the change in allele frequencies in all populations sampled at the two different times (Wang, 2001). The analysis of the historical samples also allowed assessing changes in genetic diversity and thus inbreeding throughout the last century. Part of the genetic results presented here were also used in Chapters 3 and 4.

Chapter 3 describes the contemporary genetic state and history of the endangered Floreana mockingbird. The Floreana mockingbird is the species most affected by human-induced changes which lead to the disappearance of its largest population on Floreana, and it is therefore also the most evident case where population history affected current levels of genetic diversity and inbreeding. Because of its endangered state, its planned reintroduction onto Floreana Island and its historic significance, this species is given special attention in my thesis. I describe the genetic diversity and differentiation of the two remaining contemporary satellite populations and the genetic changes over the past 100 years. The genetic analysis of two specimens from Floreana collected in 1835 provided an insight into the genetic diversity once present on Floreana and allowed a comparison with the two satellite populations. Coalescence-based modeling estimated divergence times between the three populations and showed which of the two satellite populations likely diverged first from Floreana. Based on these findings, we can make recommendations for the conservation of this species (also see *Additional Information, Conclusions, and Perspectives*).

In **Chapter 4** I combine the population genetic results with immune measures to estimate possible effects of inbreeding on immunocompetence in 13 mockingbird populations. Understanding patterns of immune responses is important to learn more about fitness and population dynamics, and to improve our knowledge on the effects that

inbreeding depression may have on disease susceptibility in wild populations. To this aim, I included a larger set of microsatellite loci to estimate population-specific Fst as a measure of long-term inbreeding (Ciofi et al., 1999, Biebach and Keller, in press) and, together with the short-term inbreeding estimate from Chapter 2, I used both Fst to assess whether inbreeding has a negative effect on innate (humoral) immunity measured as the heterophil-lymphocyte ratio, and natural antibody and complement enzyme titres. Furthermore, I included ectoparasite data to test whether more inbred populations showed higher feather louse loads than outbred populations.

The final conclusions and summary integrate the most important findings and conclusions and highlight the conservation implications for the Floreana mockingbird.

REFERENCES

ADAMO, S. A. (2004) How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour,* 68**,** 1443-1449.

APARICIO, J. M., ORTEGO, J. & CORDERO, P. J. (2007) Can a simple algebraic analysis predict markers-genome heterozygosity correlations? *Journal of Heredity,* 98**,** 93-96.

ARBOGAST, B. S., DROVETSKI, S. V., CURRY, R. L., BOAG, P. T., SEUTIN, G., GRANT, P. R., GRANT, B. R. & ANDERSON, D. J. (2006) The origin and diversification of Galapagos mockingbirds. *Evolution,* 60**,** 370-382.

BALLOUX, F., AMOS, W. & COULSON, T. (2004) Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology,* 13**,** 3021-3031.

BERRY, R. J. (2009) Hooker and islands. *Biological Journal of the Linnean Society,* 96**,** 462-481.

BIEBACH, I. & KELLER, L. F. (in press) Inbreeding in reintroduced populations: the effects of early reintroduction history and contemporary processes. *Conservation Genetics*.

BIJLSMA, R., BUNDGAARD, J. & BOEREMA, A. C. (2000) Does inbreeding affect the extinction risk of small populations? predictions from Drosophila. *Journal of Evolutionary Biology,* 13**,** 502-514.

BRANDSTROM, M. & ELLEGREN, H. (2008) Genome-wide analysis of microsatellite polymorphism in chicken circumventing the ascertainment bias. *Genome Research,* 18**,** 881-887.

CABALLERO, A. & TORO, M. A. (2000) Interrelations between effective population size and other pedigree tools for the management of conserved populations. *Genetical Research,* 75**,** 331-343.

CDF (2008) *The Reintroduction of the Floreana Mockingbird to its Island of Origin,* Puerto Ayora, Galapagos, Charles Darwin Foundation & Galapagos National Park Service.

CDF & WWF (2002) *A Biodiversity Vision for the Galapagos Islands* Puerto Ayora, Galapagos, Charles Darwin Foundation & WWF.

CHAKRABORTY, R. & NEI, M. (1977) Bottleneck effects on average heterozygosity and genetic distance with stepwise mutation model *Evolution,* 31**,** 347-356.

CIOFI, C., BEAUMONT, M. A., SWINGLAND, I. R. & BRUFORD, M. W. (1999) Genetic divergence and units for conservation in the Komodo dragon Varanus komodoensis. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 266**,** 2269-2274.

CLEGG, S. M., DEGNAN, S. M., KIKKAWA, J., MORITZ, C., ESTOUP, A. & OWENS, I. P. F. (2002) Genetic consequences of sequential founder events by an island-colonizing bird. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 8127-8132.

COLTMAN, D. W., PILKINGTON, J. G., SMITH, J. A. & PEMBERTON, J. M. (1999) Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution,* 53**,** 1259-1267.

COLTMAN, D. W. & SLATE, J. (2003) Microsatellite measures of inbreeding: A meta-analysis. *Evolution,* 57**,** 971-983.

CROW, J. F. & KIMURA, M. (1970) *An Introduction to Population Genetic Theory,* New York, Evanston and London, Harper and Row Publishers.

CURRY, R. L. (1986) Whatever happened to the Floreana mockingbird? *Noticias de Galapagos,* 43**,** 13-15.

DARWIN, C. (1839) *Voyage of the Beagle,* London, Henry Colburn.

DEWOODY, Y. D. & DEWOODY, J. A. (2005) On the estimation of genome-wide heterozygosity using molecular markers. *Journal of Heredity,* 96**,** 85-88.

ELLEGREN, H., PRIMMER, C. R. & SHELDON, B. C. (1995) Microsatellite evolution - directionality or bias. *Nature Genetics,* 11**,** 360-362.

FRANKEL, O. H. & SOULÉ, M. E. (1981) *Conservation and Evolution,* Cambridge, Cambridge University Press.

FRANKHAM, R. (1997) Do island populations have less genetic variation than mainland populations? *Heredity,* 78**,** 311-327.

FRANKLIN, I. A. (1980) Evolutionary change in small populations. IN SOULÉ, M. E. & WILCOX, B. A. (Eds.) *Conservation biology: an evolutionary-ecological perspective.,* Sinauer Associates, Sunderland, Massachusetts.

FREDRICKSON, R. J., SIMINSKI, P., WOOLF, M. & HEDRICK, P. W. (2007) Genetic rescue and inbreeding depression in Mexican wolves. *Proceedings of the Royal Society B-Biological Sciences,* 274**,** 2365-2371.

GRANT, P. R. (1998) *Evolution on Islands,* New York, Oxford University Press.

GRANT, P. R., CURRY, R. L. & GRANT, B. R. (2000) A remnant population of the Floreana mockingbird on Champion island, Galapagos. *Biological Conservation,* 92**,** 285-290.

GROOMBRIDGE, J. J., DAWSON, D. A., BURKE, T., PRYS-JONES, R., BROOKE, M. D. & SHAH, N. (2009) Evaluating the demographic history of the Seychelles kestrel (Falco araea): Genetic evidence for recovery from a population bottleneck following minimal conservation management. *Biological Conservation,* 142**,** 2250-2257.

HARRIS, M. P. (1974) *The birds of Galapagos,* New York, Houghton-Mifflin.

HEDRICK, P. W. (1995) Gene Flow and Genetic Restoration - the Florida Panther as a Case-Study. *Conservation Biology,* 9**,** 996-1007.

HOGG, J. T., FORBES, S. H., STEELE, B. M. & LUIKART, G. (2006) Genetic rescue of an insular population of large mammals. *Proceedings of the Royal Society B-Biological Sciences,* 273**,** 1491-1499.

HOULE, D. (1992) Comparing evolvability and variability of quantitative traits *Genetics,* 130**,** 195-204.

HUDSON, P. J., RIZZOLI, A., GRENFELL, B. T., HEESTERBEEK, H. & DOBSON, A. P. (2002) *The Ecology of Wildlife Diseases,* Oxford University Press.

KEIL, D., LUEBKE, R. W. & PRUETT, S. B. (2001) Quantifying the relationship between multiple immunological parameters and host resistance: Probing the limits of reductionism. *Journal of Immunology,* 167**,** 4543-4552.

KELLER, L. F., GRANT, P. R., GRANT, B. R. & PETREN, K. (2002) Environmental conditions affect the magnitude of inbreeding depression in survival of Darwin's finches. *Evolution,* 56**,** 1229-1239.

KELLER, L. F. & WALLER, D. M. (2002) Inbreeding effects in wild populations. *Trends in Ecology & Evolution,* 17**,** 230-241.

KOHLER, H., BAYRY, J., NICOLETTI, A. & KAVERI, S. V. (2003) Natural autoantibodies as tools to predict the outcome of immune response? *Scandinavian Journal of Immunology,* 58**,** 285-289.

KRISTENSEN, T. N. & SORENSEN, A. C. (2005) Inbreeding - lessons from animal breeding, evolutionary biology and conservation genetics. *Animal Science,* 80**,** 121-133.

LACY, R. C. (1997) Importance of genetic variation to the viability of mammalian populations. *Journal of Mammalogy,* 78**,** 320-335.

LAMMERS, A., KLOMP, M. E. V., NIEUWLAND, M. G. B., SAVELKOUL, H. F. J. & PARMENTIER, H. K. (2004) Adoptive transfer of natural antibodies to non-immunized chickens affects subsequent antigen-specific humoral and cellular immune responses. *Developmental and Comparative Immunology,* 28**,** 51-60.

LYNCH, M., CONERY, J. & BURGER, R. (1995) Mutation accumulation and the extinction of small populations *American Naturalist,* 146**,** 489-518.

MATSON, K. D., COHEN, A. A., KLASING, K. C., RICKLEFS, R. E. & SCHEUERLEIN, A. (2006) No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. *Proceedings of the Royal Society B-Biological Sciences,* 273**,** 815-822.

MCCALLUM, H. & DOBSON, A. (1995) Detecting Disease and Parasite Threats to Endangered Species and Ecosystems. *Trends in Ecology & Evolution,* 10**,** 190-194.

MILLER, H. C. & LAMBERT, D. M. (2004) Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). *Molecular Ecology,* 13**,** 3709-3721.

MUNDY, N. I., WINCHELL, C. S., BURR, T. & WOODRUFF, D. S. (1997) Microsatellite variation and microevolution in the critically endangered San Clemente Island loggerhead shrike (Lanius ludovicianus mearnsi). *Proceedings of the Royal Society B-Biological Sciences,* 264**,** 869-875.

NORRIS, K. & EVANS, M. R. (2000) Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology,* 11**,** 19-26.

O'BRIEN, S. J. & EVERMANN, J. F. (1988) Interactive Influence of Infectious-Disease and Genetic Diversity in Natural-Populations. *Trends in Ecology & Evolution,* 3**,** 254-259.

OCHSENBEIN, A. F. & ZINKERNAGEL, R. M. (2000) Natural antibodies and complement link innate and acquired immunity. *Immunology Today,* 21**,** 624-630.

PARMENTIER, H. K., LAMMERS, A., HOEKMAN, J. J., REILINGH, G. D., ZAANEN, I. T. A. & SAVELKOUL, H. F. J. (2004) Different levels of natural antibodies in chickens divergently selected for specific antibody responses. *Developmental and Comparative Immunology,* 28**,** 39-49.

PIMM, S. L., DOLLAR, L. & BASS, O. L. (2006) The genetic rescue of the Florida panther. *Animal Conservation,* 9**,** 115-122.

POSTMA, E. & VAN NOORDWIJK, A. J. (2005) Gene flow maintains a large genetic difference in clutch size at a small spatial scale. *Nature,* 433**,** 65-68.

PUURTINEN, M., HYTONEN, M., KNOTT, K. E., TASKINEN, J., NISSINEN, K. & KAITALA, V. (2004) The effects of mating system and genetic variability on

susceptibility to trematode parasites in a freshwater snail, Lymnaea stagnalis. *Evolution,* 58**,** 2747-2753.

ROSS-GILLESPIE, A., O'RIAIN, M. J. & KELLER, L. F. (2007) Viral epizootic reveals inbreeding depression in a habitually inbreeding mammal. *Evolution,* 61**,** 2268-2273.

SLATE, J., DAVID, P., DODDS, K. G., VEENVLIET, B. A., GLASS, B. C., BROAD, T. E. & MCEWAN, J. C. (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity,* 93**,** 255-265.

SLATKIN, M. (1985) Gene flow in natural populations. *Annual Review of Ecology and Systematics,* 16**,** 393-430.

TORO, M. A. & CABALLERO, A. (2005) Characterization and conservation of genetic diversity in subdivided populations. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 360**,** 1367-1378.

VÄLI, Ü., EINARSSON, A., WAITS, L. & ELLEGREN, H. (2008) To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? *Molecular Ecology,* 17**,** 3808-3817.

VAN RIPER, C., VAN RIPER, S. G. & HANSEN, W. R. (2002) Epizootiology and effect of avian pox on Hawaiian forest birds. *Auk,* 119**,** 929-942.

WANDELER, P., HOECK, P. E. A. & KELLER, L. F. (2007) Back to the future: museum specimens in population genetics. *Trends in Ecology & Evolution,* 22**,** 634-642.

WANG, J. L. (2001) A pseudo-likelihood method for estimating effective population size from temporally spaced samples. *Genetical Research,* 78**,** 243-257.

WATKINS, D. I., GARBER, T. L., CHEN, Z. W., TOUKATLY, G., HUGHES, A. L. & LETVIN, N. L. (1991) Unusually Limited Nucleotide-Sequence Variation of the Expressed Major Histocompatibility Complex Class-I Genes of a New-World Primate Species (Saguinus-Oedipus). *Immunogenetics,* 33**,** 79-89.

WRIGHT, S. (1931) Evolution in Mendelian populations. *Genetics,* 16**,** 0097-0159.

WRIGHT, S. (1978) *Variability within and among natural populations,* Chicago, IL, Chicago University Press.

MICROSATELLITE PRIMERS FOR THE FOUR GALÁPAGOS MOCKINGBIRD SPECIES (*MIMUS PARVULUS*, *M. MACDONALDI*, *M. MELANOTIS*, AND *M. TRIFASCIATUS*)

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Microsatellite primers for the four Galápagos mockingbird species (*Mimus parvulus*, *Mimus macdonaldi*, *Mimus melanotis* and *Mimus trifasciatus*)

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Abstract

Nineteen di- and tetranucleotide and one trinucleotide microsatellite DNA markers were isolated from the Galápagos mockingbird (*Mimus parvulus*) and tested for cross-species amplification in the other three mockingbird species in the Galápagos. In addition, primers for two microsatellite loci previously developed for *Mimus polyglottos* were redesigned to obtain shorter amplification fragments. The number of alleles per locus and species ranged from 1 to 8, and expected heterozygosity varied from 0.0 to 0.809. These microsatellite markers will be useful to study levels of inbreeding in different island populations.

Keywords: *Mimus*, multiplex PCR, *Nesomimus*

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Four endemic, allopatric mockingbird species are recognized in Galápagos (*Mimus parvulus*, *Mimus macdonaldi*, *Mimus trifasciatus* and *Mimus melanotis*). After extinction on Floreana Island, *M. trifasciatus* today only occurs on two small satellite islands and is classified as 'Critically Endangered'. To study levels of inbreeding, we designed a set of polymorphic microsatellite markers that showed similar peak intensity in all four species.

A dinucleotide- and tetranucleotide-enriched library was established separately following the FIASCO protocol (Zane *et al*. 2002). DNA from four *Mimus parvulus* individuals (two from Santiago Island, one from Santa Fé and one from Rábida) was extracted with the QIAamp DNA Mini Kit (QIAGEN) and then pooled using the same amount of DNA per individual (12.5 ng/μL each). The pooled DNA was digested with *Mse*I (New England Biolabs) and ligated to *Mse*I-AFLP-adaptors. Restricted and ligated fragments were amplified during 20 polymerase chain reaction (PCR) cycles (GeneAmp PCR System 9700, ABI) with *Mse*I-adaptor-specific primers. DNA was then hybridized with a biotinylated (AC_{17}) or tetratinylated ($GATA_8$) probe, respectively, selectively captured by magnetic beads (M-280-streptavidin-coated Dynabeads; Dynal) and nonspecific DNA removed by washing (DeWoody protocol; http://www.agriculture.purdue.edu/fnr/html/faculty/DeWoody/DeWoodyweb/pdfs/msatclngprtcl.pdf). The DNA was separated from the bead–probe complex by two denaturation steps before precipitation with sodium acetate and ethanol. Subsequently, it was re-amplified independently three times using *Mse*I-adaptor primers during 30 PCR cycles. After pooling PCR products, they were cloned by TA cloning (Invitrogen). Plasmid DNA of 340 colonies was purified (Wizard SV Plasmid DNA Purification System; Promega) and sequenced on a 3730 DNA Analyser (ABI) using BigDye Terminator v3.1 (ABI) chemistry and Better Buffer (Web Scientific). Sequence alignment and editing was carried out in BIOEDIT (Hall 1999). Twenty-four colonies contained dinucleotide, 12 tetranucleotide and two trinucleotide repeats. Primer pairs were designed for 17 dinucleotide, five tetranucleotide and one trinucleotide clones with the aim of forming different groups for PCR multiplexing using Primer 3 (Rozen & Skaletsky 2000). Primers were designed to obtain short microsatellite products (<200 bp) for later use in historical DNA samples (Wandeler *et al.* 2007). Normalized (20 ng/μL) DNA of four individuals from each species was used for initial screening for polymorphism with a M13-tailed primer method (Schuelke 2000). Fragment analyses were performed on a 3730 DNA Analyser using Gene-Scan-500 LIZ size standard (ABI) and GeneMapper v3.7 software (ABI). Only loci that amplified with similar intensity in all four species and that were polymorphic in at least one species were selected, resulting in a total of 20 microsatellite loci finally chosen, and characterized in 20 individuals of each species. DNA was extracted following the manufacturer's dried-blood spots protocol using approx. 9 mm^2 of dried blood (BioSprint 96 DNA Blood Kit; QIAGEN). DNA concentrations were standardized at

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Table 1 Characterization and summary statistics of 20 microsatellite loci genotyped in 20 individuals (100% amplification success) of each of the four mockingbird species (*Mimus parvulus*, *Mimus macdonaldi*, *Mimus melanotis* and *Mimus trifasciatus*) from Santiago, Española, San Cristóbal and Gardner-by-Floreana islands

Primer name	Repeat motif	Primer sequence (5′–3′)	Concentration (μM)	Panel	Fragment size (bp)	*Mimus parvulus* (Santiago, n = 20)				*Mimus macdonaldi* (Española, n = 20)			*Mimus melanotis* (San Cristóbal, n = 20)			*Mimus trifasciatus* (Gardner-by-Floreana, n = 20)			Accession number
						N_{AT}	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	
Nes01	$(CA)_3AA(CA)_{17}$	FAM-TCACCACAGGCAGGTAAGAAC gtttcGGGAAGAGGCAGGATAGGAC	1	B	88–112	10	8	0.650	0.684	2	0.050	0.049	2	0.400	0.320	3	0.650	0.540	FJ593004
Nes03	$(TC)_2(CT)_{14}(CA)_6$	AT550-GGAAACAGAATTCATCATCCAG gtttcTTTTCTGTTGCCCTAGTGACC	1	D	124–132	5	2	0.050	0.049	1	0.000	0.000	3	0.250	0.359	2	0.050	0.219	FJ593005
Nes04	$(GT)_3C(TGG)_2(GT)_{12}$	AT565-AACCCAACTGTGGAGGTGTG gtttcAATTCCCCCAGAAAGAATGG	0.6	C	143–151	4	3	0.150*	0.484	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	FJ593006
Nes05	$(TG)_{14}$	AT550-GGAAAAGCGATGTGAAACAAG gtttCCCAAGAGACATGCTGTGAC	0.6	A	144–178	13	8	0.900	0.725	2	0.350	0.289	3	0.250	0.301	2	0.200	0.180	FJ593007
Nes06	$(GA)_{14}$	AT565-ACCAGGAGCTATAAGGCACTC gtttcTAGGTGGAGCTCTGTGCATC	0.4	B	125–137	7	6	0.600	0.695	2	0.250	0.219	4	0.350	0.381	1	0.000	0.000	FJ593008
Nes07	$(TG)_{25}$	YYE-CCTTTTCTCTCTGTTTGATCTCTG gtttCCAGACTTCCCTTCCAATCC	1	D	173–201	12	7	0.700	0.753	4	0.550	0.556	4	0.300	0.269	2	0.100	0.255	FJ593009
Nes08	$(TC)_{13}$	AT550-GGATGTAATTGTAAAGACTCAGAGG gtttcAATTATGTTGATAAAATGCGGAGT	0.4	C	123–133	6	6	0.450	0.598	2	0.350	0.289	3	0.150	0.141	2	0.500	0.500	FJ593010
Nes10	$(CA)_4AA(CA)_4AA$ $(CA)_5CT(CA)_6$	AT550-CAATGTTCATGGCAAACTCTG gtttcGTGAGAGTGCACGTGCTTG	0.4	A	104–110	3	3	0.150	0.141	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	FJ593011
Nes11	$(TC)_{13}$	YYE-GGGGACAACTAGGGGATTTG gtttcTGGAAGCAGAGAGATTTCATCC	0.6	A	143–147	3	3	0.450	0.499	1	0.000	0.000	2	0.100	0.180	1	0.000	0.000	FJ593012
Nes12	$(GATG)_{13}$	FAM-AAGACCTGGATTTGACAGGTG gtttcTGACCACAACCCTCTGTCTG	0.6	A	122–146	7	7	0.750	0.641	4	0.800	0.671	7	0.850	0.763	4	0.600	0.656	FJ593013
Nes13	$(TGGA)_9$	FAM-AGCCTGAAGGTGGTTATTGAG gtttcTTCCTCTCAAGGATAAATGCTG	2	C	117–145	8	6	0.850	0.744	2	0.200	0.255	7	0.950	0.809	2	0.400	0.455	FJ593014
Nes14	$(GGAT)_{12}$	AT565-AATGGAGAGGGAGGGGAGA gtttcTGGCTGCTCTCTCCATATGAT	1	D	117–145	8	5	0.750	0.685	4	0.550	0.621	5	0.700	0.694	2	0.500	0.480	FJ593015
Nes15	$(TGGA)_7$	AT565-ATTACTGCAGGCAGGAAGAGG gtttcTCTGGACTTAGCACCCATCTG	0.6	A	128–160	8	3	0.350	0.301	2	0.300	0.320	7	1.000	0.796	2	0.450	0.469	FJ593016
Nes16	$(TAGA)_{10}$	AT550-GCTGTGTTTCACTCTTAgTGCAG gtttcTTAGGCTGAGAGTcTAATTCATGG	0.6	B	122–158	10	5	0.850	0.705	7	0.850	0.745	7	0.750	0.766	4	0.550	0.539	FJ593017
Nes17	$(GT)_9AT(GT)_4$ $(GAGT)_2(GA)_2$	FAM-CAATCTGTATGTAGACGTGCCTTTAG gtttcAAGTCATAAACAAGAACCTGACcTG	2	D	99–109	5	3	0.200*	0.465	1	0.000	0.000	2	0.350	0.489	1	0.000	0.000	FJ593018
Nes18	$(TG)_{11}$	AT565-GGATTGAGTAGGGTTTTGTGC gtttcATCACCAGCAGTGAAAGCAG	0.4	A	85–97	5	3	0.550	0.434	2	0.300	0.255	2	0.050	0.049	2	0.450	0.489	FJ593019
Nes19	$(CA)_{11}$	YYE-CCCTTTCCCAGATATCTTCTCTC gtttCAAATGAGGCTCTGTAAAATGC	0.6	B	110–126	6	6	0.700	0.710	1	0.000	0.000	2	0.400	0.420	1	0.000	0.000	FJ593020

Table 1 (Continued)

Primer name	Repeat motif	Primer sequence (5′–3′)	Concentration (μM)	Panel	Fragment size (bp)	*Mimus parvulus* (Santiago, n = 20)				*Mimus macdonaldi* (Española, n = 20)			*Mimus melanotis* (San Cristóbal, n = 20)			*Mimus trifasciatus* (Gardner-by-Floreana, n = 20)			Accession number
						N_{AT}	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	
Nes20	$(CAA)_{10}$	AT565-CCACAAACTCTAAATCAGTTCTGG gtttcGAGAAAAGAGACAGCATCATAACAG	0.6	C	107–118	5	5	0.550	0.671	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	FJ593021
Nes22	$(TC)_{11}$	FAM-TGTCTCACTTTTCTCAAGACCAC gtttcACCCCAAAACAGAGGAAGAG	1	D	151–167	8	7	0.600	0.761	3	0.250	0.521	3	0.500	0.626	1	0.000	0.000	FJ593022
Nes23	$(AC)_{11}$	YYE-CACTGCTTCACTTCCACAGG gtttcAACAGTAGTTGTCATGAAGGTGTAGG	1	C	104–112	4	3	0.200	0.184	1	0.000	0.000	2	0.050	0.049	1	0.000	0.000	FJ593023
MpAAT45_new	$(AAT)_{13}$†	AT565-GCTTTCCTCACCATTTCTGC ATCCCACTATAAATTTCCTGTGTA‡	2	E	155–176	7	5	0.650	0.750	2	0.300	0.320	3	0.200	0.185	3	0.550	0.491	U96326
MpAAT83_new	$(AAT)_{12}$†	YYE-CCAAATTCTTGCATGATTGAC gtttcTGTGATGCTTAAGTTTTCCCTAAG	2	E	111–144	11	8	0.700	0.703	3	0.400	0.329	7	0.650	0.595	5	0.700	0.576	U96327

Primer name, repeat motif, multiplex primer concentration, panel information of the sequenced clone, fragment size range and total number of alleles (N_{AT}) are given alongside number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities and GenBank Accession number. N_A, H_O and H_E are given for each of the four species separately. Forward primers were modified with a fluorescent label (underscore) and reverse primers with a pigtail (lower case letters). MpAAT45_new and MpAAT83_new are redesigned primers developed on the northern mockingbird (*Mimus polyglottos*) by Hughes & Deloach (1997) to reduce fragment size.

*Significant deviation from Hardy–Weinberg proportion.

†Repeats based on *Mimus polyglottos* sequences.

‡Reverse primer as published in Hughes & Deloach (1997).

20 ng/μL for further amplification (Quant-iT PicoGreen dsDNA Quantitation; Invitrogen). All 20 microsatellites were amplified in four independent multiplex reactions (GeneAmp PCR System 9700, ABI; Panel A–D; Table 1) in a total volume of 5 μL, containing 2 μL Multiplex-PCR Master Mix (QIAGEN), 0.4–2 μM of each primer (Table 1) and 1-μL template DNA. Forward primers were labelled with FAM, AT565, AT550 or YYE modifications (Microsynth AG) and a pigtail (GTTTC) was added to the reverse primers (Brownstein *et al.* 1996). Amplification profiles were as follows: 95 °C for 15 min, 28 cycles of 94 °C for 30 s, 58 °C (Panels B and C) or 59 °C (Panels A and D) for 90 s and 72 °C for 1 min, and a final extension at 60 °C for 30 min. We also redesigned primers for two markers developed in *Mimus polyglottos* (Hughes & Deloach 1997; MpAAT45_new and MpAAT83_new, Table 1) which were run in a separate Panel (E) under the same PCR conditions as Panels B and C.

To sex mockingbirds, we performed molecular sexing by amplifying the CHD-W and CHD-Z genes (Griffiths *et al.* 1998). Using the original P2 and P8 primers, we redesigned primer pairs (5′-GAGRAAYTGTGCRAAACAGG-3′, 5′-PET-GAGAYKGAGTCACTATCAGATCCAG-3′) to optimize amplification success. PCR conditions were the same as for Panels A–E, except that annealing temperature was 60 °C and primer concentrations were 1 μM, obtaining fragments of 258 and 301 bp.

All individuals were successfully genotyped for the 22 loci. Between one and eight alleles were detected in each species and observed and expected heterozygosities (GENALEX 6, Peakall & Smouse 2006) ranged from 0.0 to 1 and from 0.0 to 0.809 respectively (Table 1). No significant gametic disequilibrium (GENEPOP on the web v3.4; Raymond & Rousset 1995) was detected in pairwise comparisons across loci after Bonferroni correction for multiple comparisons (k = 22, α = 0.05), but two loci (Nes04, P = 0.0003 and Nes17, P = 0.0005) deviated significantly from Hardy–Weinberg proportions in *M. parvulus*. This can probably be explained by the presence of null alleles (estimated null allele frequencies: Nes04: 0.26, SE = 0.09; Nes17: 0.23, SE = 0.09; calculated using INEST's IIM method; Chybicki & Burczyk 2009).

The microsatellite markers described here will be useful to study the effects of limited population size and isolation on genetic diversity in different mockingbird populations, as well as the consequences of inbreeding in the two endangered *M. trifasciatus* populations.

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References

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by tag DNA polymerase: Primer modifications that facilitate genotyping. *BioTechniques*, **20**, 1004–1006.

Chybicki IJ, Burczyk J (2009) Simultaneous estimation of null alleles and inbreeding coefficients. *Journal of Heredity*, **100**, 106–113.

Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. *Molecular Ecology*, **7**, 1071–1075.

Hall TA (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Hughes CR, Deloach DM (1997) Developing microsatellites when they are rare: trinucleotide repeat loci in the northern mockingbird *Mimus polyglottos*. *Molecular Ecology*, **6**, 1099–1102.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in EXCEL. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Raymond M, Rousset F (1995) GENEPOP (version 1.2)—population-genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, **18**, 233–234.

Wandeler P, Hoeck PEA, Keller LF (2007) Back to the future: museum specimens in population genetics. *Trends in Ecology & Evolution*, **22**, 634–642.

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology*, **11**, 1–16.

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DIFFERENTIATION WITH DRIFT: A SPATIO-TEMPORAL GENETIC ANALYSIS OF GALÁPAGOS MOCKINGBIRD POPULATIONS (*MIMUS* SPP.)

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Differentiation with drift: a spatio-temporal genetic analysis of Galápagos mockingbird populations (*Mimus* spp.)

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Small and isolated island populations provide ideal systems to study the effects of limited population size, genetic drift and gene flow on genetic diversity. We assessed genetic diversity within and differentiation among 19 mockingbird populations on 15 Galápagos islands, covering all four endemic species, using 16 microsatellite loci. We tested for signs of drift and gene flow, and used historic specimens to assess genetic change over the last century and to estimate effective population sizes. Within-population genetic diversity and effective population sizes varied substantially among island populations and correlated strongly with island size, suggesting that island size serves as a good predictor for effective population size. Genetic differentiation among populations was pronounced and increased with geographical distance. A century of genetic drift did not change genetic diversity on an archipelago-wide scale, but genetic drift led to loss of genetic diversity in small populations, especially in one of the two remaining populations of the endangered Floreana mockingbird. Unlike in other Galápagos bird species such as the Darwin's finches, gene flow among mockingbird populations was low. The clear pattern of genetically distinct populations reflects the effects of genetic drift and suggests that Galápagos mockingbirds are evolving in relative isolation.

Keywords: *Nesomimus*; genetic drift; historic specimens; population differentiation

1. INTRODUCTION

Biologists have long recognized islands as ideal natural laboratories, and islands have thus contributed substantially to our understanding of evolution (Grant 1998). Two defining features of islands are their isolation and restricted land mass, both of which result in populations of limited size with clearly defined geographical boundaries. Genetic drift (the random changes of allele frequencies over generations) reduces genetic variation to an extent inversely proportional to population size (Crow & Kimura 1970); therefore, drift is expected to be pronounced in island systems (Barton 1998). Like mutation and selection, genetic drift leads to divergence among populations, whereas gene flow (migration) has a homogenizing effect (Slatkin 1985). Hence, low rates of gene flow and substantial genetic drift in perpetually small populations are the most probably explanation for the lower genetic diversity generally observed in island populations and species when compared with their mainland relatives (Frankham 1997). However, particularly in very vagile species such as birds, the effects of drift may be counteracted by gene flow. Darwin's finches in the Galápagos archipelago represent one well-known example where drift has been shown to be relatively weak due to the ubiquity of gene flow even over substantial distances (Petren *et al.* 2005). Here we examine genetic drift and diversification in another genus of birds in the Galápagos Islands, the mockingbirds, whose diversification differs substantially from that of Darwin's finches.

The mockingbirds of Galápagos played a key role in the development of Darwin's (1839) thinking on speciation. The phenotypic variation they exhibit across islands was one of Darwin's crucial observations that would ultimately lead him to propose his famous theory of evolution by natural selection (Darwin 1859). In contrast to the Darwin's finches, Galápagos mockingbird species do not occur in sympatry. Four endemic species are found in the Galápagos (Harris 1974), one of which, *Mimus parvulus*, is widespread in the archipelago occurring on most of the major islands except those occupied by the other species, whereas the other three species are very restricted in their geographical range: *Mimus trifasciatus* occurs on the islets Champion and Gardner-by-Floreana close to Floreana, *Mimus macdonaldi* on Española and Gardner-by-Española, and *Mimus melanotis* on San Cristóbal (figure 1). Unfortunately, the Floreana mockingbird (*M. trifasciatus*) today is classified as critically endangered with only 20–50 individuals left on Champion and approximately 300–500 on Gardner-by-Floreana (P. E. A. Hoeck & L. F. Keller, unpublished data) after extinction of the main population on Floreana at

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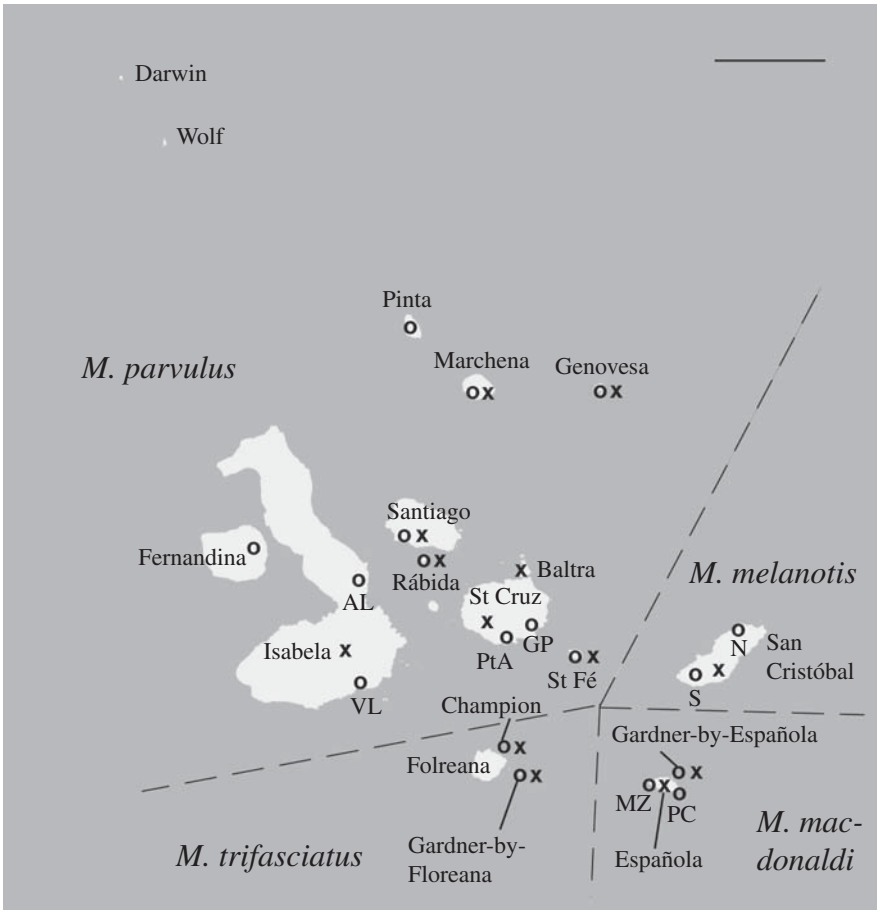

Figure 1. Distribution of the four mockingbird species in Galápagos with island names and locations from which contemporary (o) and historic (x) samples were obtained. On Isabela (AL and VL), St Cruz (PtA and GP), San Cristóbal (N and S) and Española (MZ and PC) we collected samples in two different locations (Scale bar, 50 km).

around 1880. Galápagos mockingbirds are relatively sedentary birds and hypothesized to be weak fliers as they have rarely been observed flying over water or visiting islands where there is no established population (P. R. Grant & R. L. Curry, personal communication). Because of this, migration rate is presumed to be low and genetic drift along with selection may have played a key role in the genetic structuring of mockingbird populations.

In this study, we examined genetic diversity and differentiation within and among the four mockingbird species and their populations, covering nearly their entire range and testing for evidence of genetic drift and gene flow. Using microsatellite markers, we described genetic diversity present in contemporary populations and compared it with the genetic diversity of historic populations using samples collected in the early twentieth century. We tested the prediction that genetic diversity increases with population size using island size as a proxy for population size (Frankham 1996; Petren *et al.* 2005) and assessed the role of population size in maintaining genetic diversity over time. If gene flow is limited by distance, genetic and geographical distances should be positively correlated (Wright 1943). We therefore tested for an isolation-by-distance pattern across the archipelago. Furthermore, with the help of the temporal samples, we estimated effective population size (Crow & Kimura 1970). Effective population size (Ne) has become an important measure not only in evolution but also in conservation biology because of its role in maintaining adaptive genetic diversity and evolutionary potential (Palstra & Ruzzante 2008). Finally, we compared the genetic structure among populations and species from our nuclear markers with the results published in a study on the phylogeny of the Galápagos mockingbirds based on mitochondrial DNA sequences (Arbogast *et al.* 2006). Our study provides an example of the usefulness of temporal samples to describe the change in the genetic structure between populations and species in order to disentangle temporal from spatial variation and learn more about the mechanisms underlying genetic diversification in small and isolated populations.

2. MATERIAL AND METHODS

(a) *Sample collection*

(i) *Contemporary samples*

Blood samples from a total of 543 individuals from 14 islands in the Galápagos were collected between 2003 and 2008 (figure 1). We sampled on all islands inhabited by mockingbirds, except for two small, remote islands in the northwest of the archipelago (Darwin and Wolf). In order to estimate within-island differentiation, we obtained samples from two separate locations on three large islands (Isabela: AL and VL;

Table 1. Populations studied and number of successfully genotyped samples from each population (*n*). Measures of genetic variation based on 16 microsatellite loci: Na: number of alleles, AR: average allelic richness, He: expected heterozygosity, Ho: observed heterozygosity, P: polymorphism. Island size is shown in hectares and island isolation was calculated as nearest shore-to-shore distances from all other islands (km). Island location was assigned as central (c) or peripheral (p) based on the position of the island within the species range of *M. parvulus*. Island age is shown in million years.

	n	total Na	mean Na	AR	He	Ho	P	island size (ha)	island size (ln)	island isolation	island location	island age (10^6 years)
Baltra-CAS	18	60	3.75	3.25	0.560	0.488	0.94	2619.6	7.87	68.34	n.a	1.1
Champion	48	21	1.31	1.20	0.072	0.082	0.25	9.5	2.25	87.69	n.a.	1.5
Champion-CAS	11	20	1.25	1.25	0.118	0.084	0.25	9.5	2.25	87.69	n.a.	1.5
Española, MZ	29	35	2.19	1.86	0.225	0.220	0.50	6048.0	8.71	111.15	n.a.	3
Española, PC	58	40	2.50	1.81	0.211	0.222	0.44	6048.0	8.71	111.15	n.a.	3
Española-CAS	25	40	2.50	2.05	0.279	0.215	0.69	6048.0	8.71	111.15	n.a.	3
Fernandina	24	66	4.13	2.93	0.438	0.419	0.81	64248.0	11.07	122.84	p	0.035
Gardner-by-Española	10	27	1.69	1.59	0.167	0.194	0.44	58.0	4.06	116.47	n.a.	3
Gardner-by-Española-CAS	12	24	1.50	1.45	0.174	0.177	0.38	58.0	4.06	116.47	n.a.	3
Gardner-by-Floreana	69	34	2.13	1.77	0.261	0.250	0.56	81.2	4.40	93.59	n.a.	1.5
Gardner-by-Floreana-CAS	27	38	2.38	1.95	0.276	0.234	0.50	81.2	4.40	93.59	n.a.	1.5
Genovesa	37	37	2.31	1.95	0.290	0.296	0.63	1410.8	7.25	120.09	p	0.3
Genovesa-CAS	29	34	2.13	1.76	0.221	0.197	0.56	1410.8	7.25	120.09	p	0.3
Isabela, AL	32	72	4.50	2.97	0.430	0.436	0.81	458812.0	13.04	69.37	c	0.5
Isabela, VL	30	73	4.56	3.24	0.486	0.475	0.81	458812.0	13.04	69.37	c	0.5
Isabela-CAS	27	75	4.69	3.18	0.476	0.417	0.88	458812.0	13.04	69.37	c	0.5
Marchena	38	55	3.44	2.63	0.455	0.451	0.88	12996.0	9.47	107.75	p	0.6
Marchena-CAS	24	52	3.25	2.74	0.470	0.429	0.88	12996.0	9.47	107.75	p	0.6
Pinta	27	46	2.88	2.30	0.370	0.359	0.69	5940.0	8.69	134.41	p	0.7
Rábida	21	50	3.13	2.56	0.423	0.461	0.69	499.3	6.21	78.05	c	1.3
Rábida-CAS	27	49	3.06	2.45	0.410	0.402	0.75	499.3	6.21	78.05	c	1.3
San Cristóbal, N	17	46	2.88	2.46	0.353	0.353	0.63	55808.6	10.93	101.23	n.a.	2.4
San Cristóbal, S	20	54	3.38	2.67	0.378	0.381	0.63	55808.6	10.93	101.23	n.a.	2.4
San Cristóbal-CAS	27	57	3.56	2.70	0.390	0.348	0.63	55808.6	10.93	101.23	n.a.	2.4
Santiago	27	82	5.13	3.50	0.534	0.502	1.00	58465.0	10.98	69.29	c	0.8
Santiago-CAS	29	84	5.25	3.56	0.521	0.504	0.88	58465.0	10.98	69.29	c	0.8
St Cruz, GP	22	70	4.38	3.38	0.579	0.586	1.00	98555.0	11.50	53.24	c	1.1
St Cruz, PtA	13	66	4.13	3.46	0.594	0.582	0.94	98555.0	11.50	53.24	c	1.1
St Cruz-CAS	27	84	5.25	3.62	0.568	0.453	1.00	98555.0	11.50	53.24	c	1.1
St Fé	21	33	2.06	1.81	0.255	0.235	0.56	2413.0	7.79	73.64	p	2.9
St Fé-CAS	25	35	2.19	1.72	0.218	0.148	0.56	2413.0	7.79	73.64	p	2.9
total/average	851	143	3.18	2.47	0.366	0.348	0.69					

St Cruz: GP and PtA; San Cristóbal: N and S) and one medium-sized island (Española: PC and MZ). On Champion we managed to sample the entire population except one individual. Sample sizes from the different locations varied between 10 and 69 individuals (mean: 30 individuals; table 1).

(ii) *Historic samples*

Historic tissue samples from 349 specimens from 13 islands were obtained from the museum collection of the California Academy of Sciences (CAS). The majority of the specimens were collected during the CAS expedition to the Galápagos in the years 1905 and 1906 (called 1906 below), with a few samples collected in 1899. We were thus able to obtain both contemporary and historic samples from 12 islands, contemporary samples only from two (Fernandina and Pinta) and historic samples only from one island (Baltra; figure 1). The Baltra mockingbird population went extinct during or after World War II (Curry 1986); however, the historic samples are interesting to determine the former population's genetic relationship to the surrounding populations. Sample sizes for the historic populations (referred to as *CAS*-populations) ranged from 11 to 29 specimens per island (mean: 24; table 1).

(b) *DNA extraction and microsatellite analysis*

(i) *Contemporary samples*

Blood samples were collected on filter paper after a small puncture of the wing vein of live birds. Extraction and PCR were performed using previously published methods (Hoeck *et al.* in press *b*). Concentrations of DNA extracts were standardized at 20 ng μl^{-1} (Quant-iT PicoGreen dsDNA Quantitation, Invitrogen) and the following 17 microsatellite loci were amplified: MpAAT26, Nes01, Nes03, Nes04, Nes06, Nes10, Nes12, Nes13, Nes14,

Nes15, Nes16, Nes17, Nes18, Nes19, Nes20, Nes22 and Nes23. Except for MpAAT26 which was developed in *Mimus polyglottos* by Hughes & Deloach (1997), all microsatellite loci were previously designed in our laboratory (Hoeck *et al.* in press *b*) with the aim of obtaining short microsatellite products (less than 200 bp) for amplification in highly fragmented, low quality DNA. Microsatellites were amplified in four independent multiplex reactions as described in Hoeck *et al.* (in press *b*). MpAAT26 was amplified separately under the same conditions as markers in multiplex reactions B and C. Fragment analyses were performed on a 3730 DNA Analyser using GeneScan-500 LIZ size standard (ABI) and GENEMAPPER v. 4 software (ABI) followed by manual proofreading of genotypes. To estimate the frequency of genotyping error rates, six per cent of the contemporary samples were amplified and genotyped a second time at each locus.

(ii) *Historic samples*

Small toe pad samples (approx. 4 mm^2 in size) were collected from the historic specimens and half of each sample was used for DNA extraction using QIAamp DNA Micro kit (QIAGEN) following the manufacturer's tissue protocol. Negative controls were included and all work with historic samples was carried out in a dedicated historic DNA laboratory where no contemporary mockingbird DNA had ever been present. The laboratory had an independent air-handling system, was under positive air displacement and was irradiated with UV light to destroy DNA following each laboratory session. The DNA concentration in the historic samples was measured through quantitative PCR (QPCR) using SYBR Green I detection format (Roche Diagnostics, Switzerland) by amplifying part of the 7 intron of the fibrinogen gene β-subunit (Prychitko & Moore 1997). Using the FIB-BI7U and FIB-BI7L primers developed by Prychitko & Moore (1997), we sequenced *M. trifasciatus* DNA to design two new primers for QPCR, NesFib7F (5′-CTGGATGCAATAGTCAGAGACTG-3′) and NesFib7R (5′-CCTGCCTCTTTCTTCAGGAC-3′), in order to reduce the amplicon length to 104 bp. The ABI 7500 Fast Real-Time PCR System (Applied Biosystems) was used for QPCR amplification and detection. Negative controls were included in the experimental runs and 1–2 replicates were done for each historic sample. QPCR was prepared in a 20 µl reaction volume containing 10 µl of FastStart Universal SYBR Green Master (ROX), 300 nM of each primer and 2 µl template DNA following the operator's manual for PCR conditions. DNA concentrations were determined using a standard curve consisting of 11 dilutions (of modern *M. trifasciatus* DNA) ranging from 0.005 to 20 ng μl^{-1}.

PCR amplification of the 17 microsatellites was carried out as described in Hoeck *et al.* (in press *b*) with the exception that the total reaction volume of 5 µl contained 2.5 µl Multiplex PCR Master Mix (QIAGEN) and 2 µl of template historic DNA. Negative controls were included to monitor potential contamination. PCR conditions were changed slightly from the protocol described in Hoeck *et al.* (in press *b*), with an initial denaturation step of only 12 min followed by 38 cycles of amplification at 59°C for all four panels. To assure reliable genotyping of the historic samples, PCR amplification was replicated four times for each sample at each locus. This should be sufficient as 2–3 replicates have previously been shown to accurately score the genotype in 99 per cent of sample- and locus-combinations in museum samples containing reasonable amounts of DNA (Sefc *et al.* 2003). Fragment analyses and genotyping were done as described above. The software GIMLET (Valiere 2002) was used to determine drop-out and genotyping error rates per locus as well as consensus genotypes for each sample based on the four replicates.

(c) *Diversity within populations*

Deviations from Hardy–Weinberg equilibrium (HWE) for each locus were tested with allele randomizations within samples (1000 permutations per test) and overall samples (10 000 permutations) using FSTAT 2.9.3.1 package (Goudet 2001) and Bonferroni corrections. Genotypic equilibrium between all pairs of loci in each population was tested using G-statistics with Bonferroni corrections (FSTAT; 84 000 permutations). To describe within-population genetic diversity, we calculated standard parameters such as mean number of alleles (Na), allelic richness (AR, standardized to the smallest sample size), observed (Ho) and expected heterozygosity (He) and polymorphism (P) in FSTAT and GENETIX v 4.05 (Belkhir *et al.* 2004).

(i) *Contemporary populations*

We tested for the predicted positive correlation between genetic diversity and population size using He, AR and P as estimators of genetic diversity. Because no empirical information on current population sizes was available except for the two *M. trifasciatus* populations, we used island size as a surrogate for population size (Frankham 1996). In a multiple regression analysis, we entered island size as an explanatory variable and the within-population indices of genetic diversity as dependent variables in separate analyses. As more isolated islands are less likely to receive gene flow than islands situated at the centre of the archipelago and older island populations might have lost more genetic diversity due to drift and reduced gene flow, we entered island isolation and island age as further explanatory variables. Average isolation for each island was calculated by adding up nearest shore-to-shore distances to all other islands and dividing the sum by the total number of islands minus one (Hamilton & Rubinoff 1967). For island age, we used the youngest age estimate for each island (D. Geist 2005–2008, unpublished data). The three explanatory variables (island size, isolation and age) were not correlated (all $r^2 < 0.18$). Island size was ln-transformed, but all other variables and their residuals showed no significant deviation from normality.

(ii) *Temporal change within populations*

We performed a Wilcoxon signed-rank test with the variables He, AR and P to test whether these within-population indices of genetic diversity changed significantly over the last century. Based on the assumption that genetic drift is stronger in smaller and more isolated populations, we also investigated whether change in genetic diversity was dependent on island size or isolation. To this end, we performed a multiple regression analysis using size and isolation as explanatory variables and the relative change in He, AR and P between the historic and contemporary populations as dependent variables (i.e. $1-\mathrm{He}_{\mathrm{Contemporary}}/\mathrm{He}_{\mathrm{CAS}}$, etc). To quantify the change in gene frequencies within each island since 1906 (i.e. temporal differentiation), we calculated Weir & Cockerham's (1984) estimator τ for Wright's F_{ST} (GENEPOP on the web v. 3.4; Raymond & Rousset 1995) for each CAS-contemporary population pair (called 'temporal F_{ST}' below) and related it to island size or isolation, respectively, in a linear regression analysis. We chose F_{ST} to estimate temporal differentiation within islands because of the relatively small time scale involved (approx. 25 generations assuming a generation time of 4 years; Grant *et al.* 2000). Over such short time scales drift is the dominant process creating local differentiation and the effects of mutation are minimal (Slatkin 1995).

(iii) *Effective population size*

In the absence of migration, selection and mutation, effective population size can be estimated using temporal changes in allele frequencies (Wang 2001). We used our temporal dataset and the Bayesian coalescent-based method implemented in the program CoNe (Anderson 2005) to calculate the variance effective population size (Ne) for each island population, setting the time between the two sampling periods to 25 generations, the likelihood range for Ne between 2 and 20 000 in steps of 5, and using 1000 Monte Carlo replications.

(d) *Differentiation among populations and species*

(i) *Pair-wise population differentiation*

Differentiation over all loci for all contemporary population pairs was estimated using Nei's standard genetic distance Ds (Nei 1972) calculated in POPULATIONS v. 1.2.30 (Langella 2000). We chose Ds because it allows for mutation and increases more linearly with time than F_{ST} when considering large time scales and, hence, is a more accurate estimator when estimating evolutionary times (Takezaki & Nei 1996). Furthermore, Ds does not assume a specific mutation model and has been shown to perform well with microsatellite data (e.g. Takezaki & Nei 1996; Paetkau *et al.* 1997; Petren *et al.* 1999). F_{ST} (Weir & Cockerham 1984) was calculated for comparison. Including only populations of *M. parvulus* to avoid species bias we also tested whether overall differentiation between peripheral islands (table 1) was higher than between centrally located islands in the archipelago. We tested for isolation-by-distance by contrasting geographical distances and multi-locus Ds-values between all contemporary population pairs of *M. parvulus* and, separately, also between all populations of all four species, using a series of Mantel tests (1000 permutations; Raymond & Rousset 1995). Geographical distance was measured as the logarithm of each island's nearest shore-to-shore distance from the other islands in the archipelago (Hamilton & Rubinoff 1967; Google Earth v 5.0, Google Inc.).

(ii) *Genetic affinities among species and populations*

Genetic affinities among contemporary species and populations were described with a factorial correspondence analysis (FCA) on multilocus genotypes using GENETIX v 4.05 (Belkhir *et al.* 2004). FCA displays the genetic differences among populations in a two-dimensional graphical space. Genetic distances among populations were also assessed by building an evolutionary tree based on Nei's Ds using UPGMA and performing 1000 bootstrap resamplings among loci with POPULATIONS v. 1.2.30 (Langella 2000).

All statistical analyses were done using JMP v. 8 (SAS Institute Inc., Cary, NC).

3. RESULTS

(a) *Genotyping*

Amplification and genotyping of the contemporary samples was very successful reaching nearly 100 per cent, with only one locus not amplifying in a single individual. Genotyping error and dropout rates in the contemporary samples were below 0.1 per cent. Not surprisingly, amplification success for the historic samples was lower, most probably due to the much lower DNA concentrations ($0.01-254$ pg μl^{-1} with an average of 28 pg μl^{-1}) and lower DNA quality of historic samples in general (Wandeler *et al.* 2007). On average, 78 per cent of the PCR reactions with the historic samples resulted in successful amplification (across individuals and loci) and the combined allelic dropout and false allele rates of all four replicates of the historic samples were seven per cent (s.d. = 0.08) and 2.8 per cent (s.d. = 0.04), respectively. Consensus genotypes were crosschecked for reliability by hand. Additionally, blank negative controls confirmed that cross-contamination was negligible. Forty-two individuals from the CAS collection amplified successfully for less than 10 loci and were therefore excluded from all further analyses.

(b) *Diversity within populations*

Fourteen of 17 loci were in HWE in all populations, and no genotypic disequilibrium was detected for any pairs of loci in any population. Nes22 significantly deviated from HWE in various populations (San Cristóbal-CAS, Santiago-CAS, Marchena and Marchena-CAS) and was therefore excluded from all further analyses. Nes16 and Nes04 deviated significantly from HWE in a single population each (Española-CAS and Santiago, respectively) showing an excess of homozygotes. If null alleles were the cause of these deviations, we would expect to find other populations out of HWE for these loci as well. Therefore, these deviations most probably reflect substructure within the populations although an

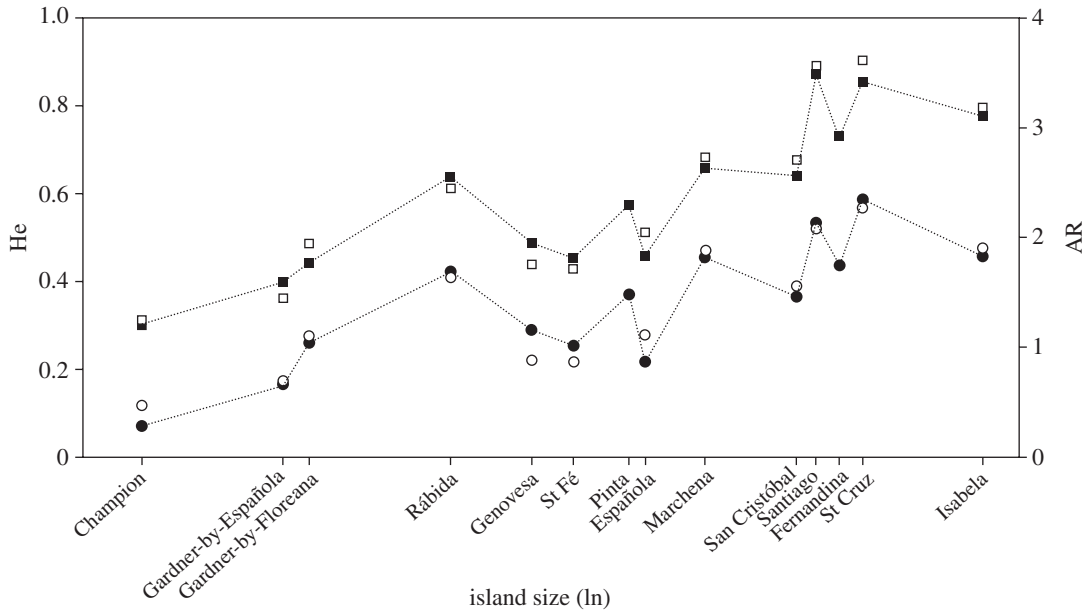

Figure 2. Expected heterozygosity (He) and allelic richness (AR) of contemporary and historic populations as a function of the natural logarithm of island size (in ha). To improve visual representation, points that overlapped were slightly moved (filled circle, contemporary He; open circle, historic He; filled square, contemporary AR; open square, historic AR).

overall Wahlund effect is unlikely with only one locus out of HWE.

We identified a total of 143 alleles across all 16 remaining loci (table 1), with individual loci having between 3 and 19 alleles (average: 8.9 alleles) and individual populations having between 1 and 11 alleles per locus (average: 3.1 alleles). Genetic diversity, measured as mean number of alleles (Na), allelic richness (AR) and heterozygosities (He and Ho), varied greatly between different populations (table 1 and figure 2). Ho ranged from 0.08 to 0.59 (mean: 0.35) and correlated strongly ($r^2 = 0.95$, $p < 0.0001$) with He (range: 0.07–0.59; mean: 0.37). Average AR ranged between 1.2 and 3.6 and P between 25 and 100 per cent. Na and AR correlated strongly ($r^2 = 0.95$, $p < 0.0001$) indicating that both measures are equally suited to describe genetic diversity. Overall, Champion and Gardner-by-Española showed the lowest and Santiago and St Cruz the highest estimates of genetic diversity (table 1 and figure 2).

(i) *Contemporary populations*

All measures of genetic diversity were significantly related to island size (He: $F_{1,14} = 15.9$, $b = 0.03 \pm 0.01$, $p = 0.001$; AR: $F_{1,14} = 33.4$, $b = 0.14 \pm 0.05$, $p < 0.0001$; P: $F_{1,14} = 12.1$, $b = 0.04 \pm 0.01$, $p = 0.004$; figure 2) and island age (He: $F_{1,14} = 6.3$, $b = -0.05 \pm 0.02$, $p = 0.025$; AR: $F_{1,14} = 8.7$, $b = -0.21 \pm 0.07$, $p = 0.011$; P: $F_{1,14} = 6.4$, $b = -0.08 \pm 0.03$, $p = 0.024$) but not isolation (He: $F_{1,14} = 3.1$, $b = -0.0014 \pm 0.0008$, $p = 0.101$; P: $F_{1,14} = 1.7$, $b = -0.002 \pm 0.001$, $p = 0.22$) except for AR ($F_{1,14} = 5.6$, $b = -0.007 \pm 0.003$, $p = 0.033$). The results showed an overall pattern of genetic diversity increasing significantly with island size and decreasing with island age and, at least for AR, also with isolation.

(ii) *Temporal change within populations*

Overall, within-population genetic diversity estimates of the CAS-populations were not significantly different from the contemporary populations (Wilcoxon: He, $p = 0.67$; AR, $p = 0.42$; P, $p = 1.0$), indicating that archipelago-wide genetic diversity did not change significantly since 1906 (figure 2). Also, we detected no significant relationship between island size or isolation and change in He, AR or P over the last century (all p-values above 0.34). However, when studying the genetic diversity estimates for individual populations, it becomes evident that changes did occur in some cases and some contemporary populations have individually lost or gained genetic diversity: the Champion population lost 39 per cent, Española 22 per cent and the two Gardners 4–5% of their expected heterozygosity during the last 100 years, whereas He for the populations on Genovesa and St Fé increased by 32 and 17 per cent, respectively (table 1 and figure 2).

Using temporal F_{ST}, which quantifies the change in gene frequencies within each island since 1906, we found that the degree of genetic differentiation was significantly negatively correlated with island size ($r^2 = 0.93$, $p < 0.0001$) but not island isolation ($r^2 = 0.14$, $p = 0.23$). Genetic differentiation was stronger in smaller than in larger populations during the last 100 years as expected from genetic drift (figure 3*a*; see the electronic supplementary material).

(iii) *Effective population size*

We were able to estimate effective population size for all 12 populations for which we had temporal samples. The lowest maximum likelihood Ne estimate was 43 individuals for Champion, and the highest was 1591 individuals for Isabela, reflecting the smallest and largest islands investigated (see the electronic

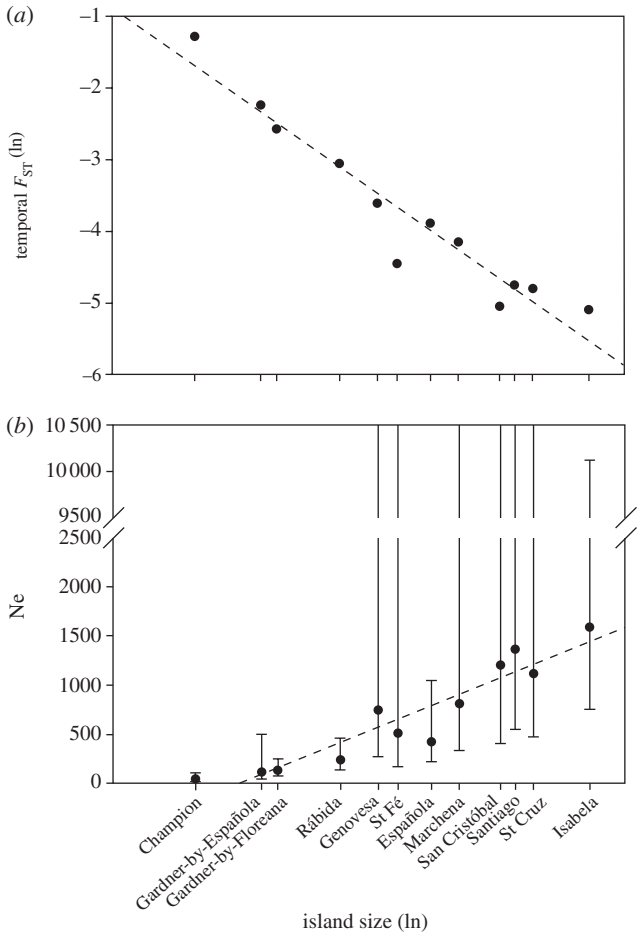

Figure 3. (*a*) Temporal F_{ST} between contemporary and historic populations as a function of island size (in ha; log scale). Differentiation clearly increased more strongly in smaller populations as shown by the dotted regression line ($F_{ST} = -0.899 - 0.354 * \log(\text{island size})$). Overlapping data points from islands with similar size were slightly modified to improve visual representation. (*b*) Estimates of effective population size (Ne) with lower and, where available, upper estimation limits plotted against island size (in ha; log scale). The dotted line shows the linear regression line ($\text{Ne} = -507.07 + 148.94 * \log(\text{island size})$). A strong positive linear correlation was also found when both Ne and island size were ln-transformed ($r^2 = 0.92$, $p < 0.0001$).

supplementary material). In six cases no upper confidence interval could be calculated, resulting in an infinite upper support limit. Ne estimates were strongly positively correlated with island size ($r^2 = 0.88$, $p < 0.0001$; figure 3*b*).

(c) *Differentiation among populations and species*

(i) *Pair-wise population differentiation*

Pair-wise differentiation (Ds) between all contemporary populations and species from different islands ranged from 0.004 to 1.988 (see the electronic supplementary material). Pair-wise Ds and F_{ST} correlated strongly ($r^2 = 0.75$, $p < 0.0001$; see the electronic supplementary material) and qualitatively provided the same results. In general, the highest values occurred between populations belonging to different mockingbird species (mean Ds = 0.86 ± s.d. 0.37). Ds-values were lower for within-species comparisons (mean Ds = 0.37 ± s.d. 0.19) and lowest for comparisons between localities within islands (mean Ds = 0.04 ± s.d. 0.03). However, differentiation between the Alcedo (AL) population on Isabela and the population on neighbouring Fernandina (Ds = 0.024) was lower than the differentiation between the two sites on Isabela (AL and VL, Ds = 0.042). Also, differentiation between Isabela VL and Fernandina (Ds = 0.037) was slightly lower than between Isabela AL and VL. We found that overall differentiation between *M. parvulus* populations was higher among peripheral than among centrally located islands (mean peripheral Ds = 0.54 ± s.d. 0.12 versus mean central Ds = 0.32 ± s.d. 0.17). The Mantel test showed a highly significant relationship between genetic differentiation and geographical distance for pairs of *M. parvulus* populations ($r^2 = 0.22$, $p = 0.001$) and also across populations of all four species ($r^2 = 0.16$, $p = 0.001$). Thus, isolation-by-distance, i.e. an increase in genetic differentiation with increasing between-island distances was evident.

(ii) *Genetic affinities among species and populations*

Genetic differences among contemporary populations based on an FCA analysis revealed three main clusters, with *M. trifasciatus* most clearly differentiated from the other species (figure 4), all populations of *M. parvulus* forming a second cluster and *M. macdonaldi* and *M. melanotis* together forming a third cluster. Similar population relationships were found in the UPGMA tree, which showed all populations of *M. parvulus* separated from the other three species, populations of *M. macdonaldi* most closely together with *M. melanotis* and *M. trifasciatus* forming a separate branch (figure 5). As we were unable to root our tree due to the lack of microsatellite data from a related species, we cannot show the evolutionary position of the clusters.

4. DISCUSSION

Our analyses revealed that genetic drift has strongly shaped the distribution of genetic variance within and between mockingbird populations and species. Furthermore, on an archipelago-wide scale genetic diversity did not change over the past 100 years, suggesting that overall the mockingbird populations in Galápagos are in or close to migration-drift equilibrium at neutral loci.

(a) *Diversity within populations*

Levels of genetic diversity varied greatly among mockingbird populations (table 1). Overall, the amount of genetic diversity was lower in *M. trifasciatus* and *M. macdonaldi* than in *M. melanotis* and, especially, *M. parvulus* (table 1 and figure 2). These results reflect the wide distribution of *M. parvulus* in the archipelago (figure 1) and are in line with the general finding of increased genetic diversity on larger islands. Additionally, the lower genetic diversity in the three range-restricted species might also reflect their occurrence on older and more isolated islands. Island age, and to a lesser degree, island isolation were also

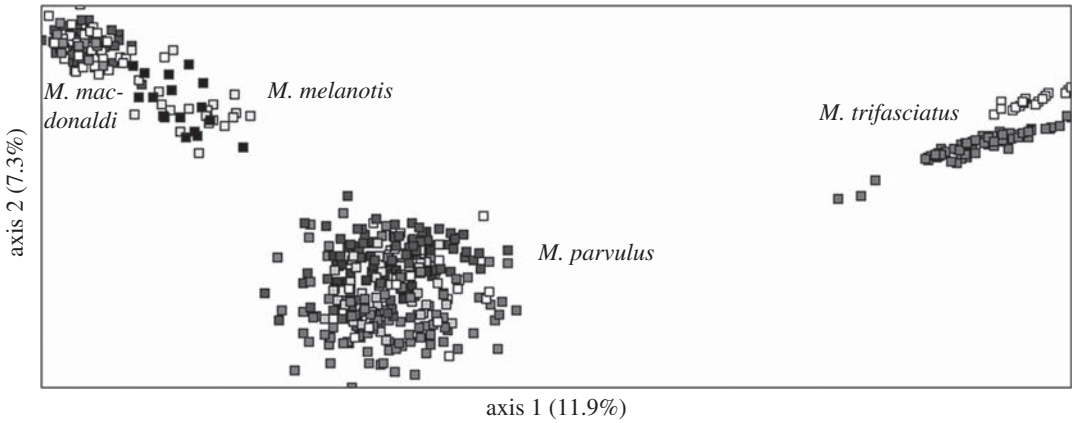

Figure 4. A two-dimensional diagram representing the relationships between the four mockingbird species based on a factorial correspondence analysis on multilocus genotypes. Only the first two axes are represented with the percentage of variance explained by the axes in parentheses.

found to affect levels of genetic diversity. The positive relationship between genetic diversity and island size and the negative relationship with island isolation and age suggest that gene flow is generally limited. The pattern found here is unlikely due to the older age and smaller size of peripheral islands because we found no significant correlation between the explanatory variable island size, isolation and age. However, the effects of gene flow seem to depend on the neighbouring islands. Smaller islands that are adjacent to islands with high levels of genetic diversity harboured considerably higher levels of genetic diversity than expected for their size (e.g. Rábida and Baltra; figure 2 and table 1). On the other hand, although close to a larger island, Gardner-by-Española did not have elevated levels of genetic diversity presumably because Española itself showed low levels of genetic diversity.

Levels of genetic diversity in the mockingbirds were much lower than those found in other Galápagos bird species such as Darwin's finches (Petren *et al.* 2005) or the Galápagos dove (Santiago-Alarcon *et al.* 2006). This could be due to the mockingbirds' relatively sedentary behaviour in comparison to the much more vagile finches and doves. In addition, mockingbirds may have smaller effective population sizes (see below). Comparing levels of genetic diversity at microsatellite loci across species is often hampered by ascertainment bias resulting from the selection for polymorphism during marker development (Brandstrom & Ellegren 2008). In cross-species comparisons, this ascertainment bias can lead to artifactual differences because the loci will be more polymorphic in the species in which the microsatellite loci were developed (e.g. Ellegren *et al.* 1995). In designing our microsatellite loci, we deliberately avoided creating such ascertainment bias among the four mockingbird species in Galápagos by including all loci that were polymorphic in at least one of the species in our panels (Hoeck *et al.* in press *b*). However, when comparing the mockingbirds to other bird species in Galápagos we cannot rule out that ascertainment bias affects our conclusions.

Overall there was little difference in estimates of genetic diversity between the CAS- and contemporary populations. However, change in diversity was considerable in a few cases (figure 2): the Champion and Española populations lost a substantial amount of He, clearly showing that these populations are individually not in drift-gene-flow equilibrium. Interestingly, levels of He were higher in the contemporary populations on Genovesa and St Fé than in the historic ones, a result that is less intuitive. We can rule out genotyping errors in the CAS samples as a major cause of these findings because estimates of allelic dropout rates among the historic samples on these two islands were very small. It seems more probably that these increases were due to biased sampling in the field, genetic drift or immigration. The occurrence of immigration is a possible explanation for the contrasting nuclear and mitochondrial DNA pattern found in the Genovesa population (see discussion below).

Overall, allele frequency distributions changed more in smaller than in larger populations: within-island genetic differentiation between 1906 and the present was much stronger on smaller islands (figure 3*a*) indicating more pronounced genetic drift in small populations. However, despite these clear signals of genetic drift, absolute levels of genetic diversity changed remarkably little on an archipelago-wide scale between 1906 and today. This is perhaps not so surprising since it simply implies that overall Galápagos mockingbird populations are in migration-drift equilibrium at neutral loci. Two other studies of undisturbed island populations (Taylor *et al.* 2007; Pertoldi *et al.* 2008) have similarly shown stability in genetic diversity over time, suggesting that our finding of migration-drift equilibrium at neutral loci may not be unusual for islands without major anthropogenic disturbances.

Estimates of effective population size (Ne) were strongly related to island size, suggesting that the latter is a reliable estimator of Ne (figure 3*b*). On the two islands where we had data on census sizes and estimates of Ne, the two were in reasonable agreement (Champion: Nc = 20–50, Ne = 43 (95% CI: 17–107), Gardner-by-Floreana: Nc ≈ 300–500, Ne = 133 (95% CI: 75–245)). The strong correlation between Ne and island size indicates that mockingbird habitats are quite equally distributed throughout the

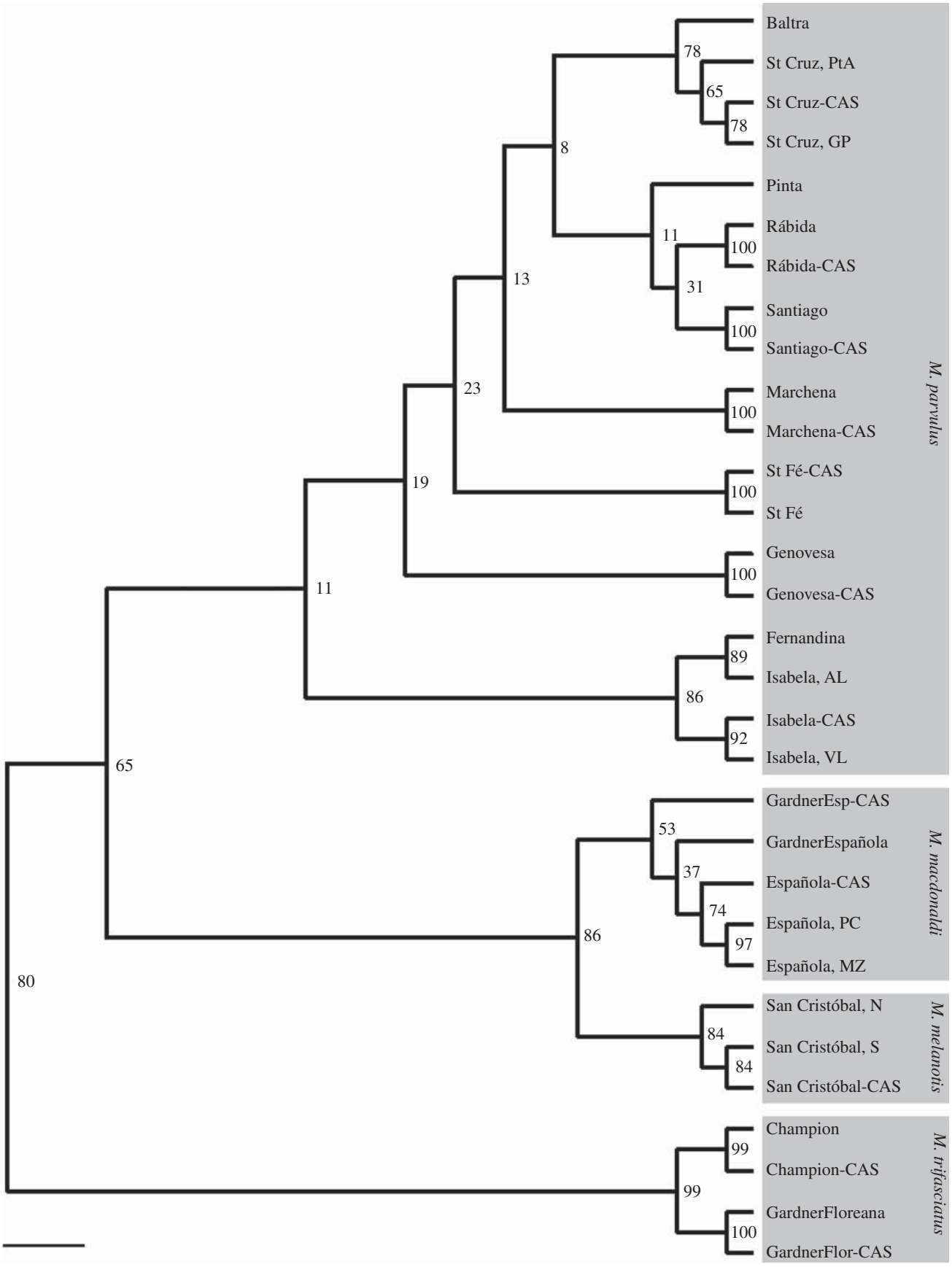

Figure 5. Unrooted UPGMA tree based on Nei's Ds with bootstrap values over loci. The shaded areas behind the population names represent the four different *Mimus* species. Scale bar, 0.1.

different islands despite the vast areas of lava occurring on some of the larger islands. The more humid transitional zones at higher altitudes that only occur on these larger islands and provide good mockingbird habitat might compensate for the lack of habitat in lava field areas. In addition, the fact that the relationship

between island size and Ne was linear (figure 3*b*) suggests that subdivision within island was not very strong, a view supported by the significant but small degrees of differentiation between populations on the same island (see the electronic supplementary material).

Ne estimates found in this study ranged from 47 to 1591 with a mean of 692 (see the electronic supplementary material). Thus, all but five mockingbird populations in Galápagos have Ne values at levels currently thought to represent viable populations (Ne = 500–5000; e.g. Franklin & Frankham 1998). On smaller islands such as Rábida and Gardner-by-Española Ne estimates are lower, most probably due to restricted territorial space, but occasional gene flow from the adjacent larger populations probably contributes to maintaining genetic diversity. In contrast, since the extinction of the population on Floreana, this is no longer possible for the two *M. trifasciatus* populations. As a consequence, Champion in particular experienced low Ne and loss of genetic diversity over the past 100 years.

(b) *Differentiation among populations and species*

Genetic differentiation among contemporary populations was high for most population pairs (see the electronic supplementary material). As expected, we found differentiation to be strongest between populations belonging to different species and lowest between populations of the same species on adjacent islands. The low differentiation found between the two *M. macdonaldi* populations suggests gene flow between Española and its satellite population. Levels of differentiation between the two *M. trifasciatus* populations were about 10 times higher, of a magnitude comparable to those of between-species comparisons. This pattern can be explained by the very low population size on Champion resulting in strong genetic drift, and by a lack of gene flow between Gardner and Champion since the extinction of the population on Floreana (Hoeck *et al.* in press *a*). In addition, the low percentage of polymorphic loci on Champion may also have contributed to the high F_{ST} estimates (Hedrick 1999). We also measured within-island differentiation on four islands and found that it was lower than between-island differentiation for three of them. The higher differentiation within Isabela than among Isabela and Fernandina (see the electronic supplementary material) suggests that mockingbird dispersal is affected more by geographical proximity than separation by water, a view also reflected in the general isolation-by-distance pattern detected in this study. However, the substantial within-island genetic diversity on Isabela and Fernandina could also contribute to the low Ds (F_{ST}) values (Hedrick 1999).

We found significant isolation-by-distance among populations of all four species and also within *M. parvulus*. Under isolation-by-distance, the most striking differences are expected to occur between peripheral populations, a pattern that was corroborated by the higher pair-wise Ds-values between peripheral mockingbird populations compared with central ones. Stronger genetic differentiation between peripheral and geographically more distant populations was also detected in cactus and ground finches (Petren *et al.* 2005) but not in warbler finches where dispersal was limited by habitat similarity (Tonnis *et al.* 2005).

As with genetic diversity, overall levels of genetic differentiation among mockingbird populations contrast with that found for other species in the Galápagos, which show much lower inter-population differentiation (e.g. Ciofi *et al.* 2002; Petren *et al.* 2005; Santiago-Alarcon *et al.* 2006). However, our findings are comparable to levels of differentiation found between Galápagos hawk (Bollmer *et al.* 2005) or land iguana populations (Tzika *et al.* 2008), for example, reflecting the effects of pronounced genetic drift and restricted gene flow.

Genetic affinities among species. Our multilocus microsatellite data revealed three major clusters, with *M. macdonaldi* and *M. melanotis* grouping closely together, all *M. parvulus* populations forming a second group, and *M. trifasciatus*, the most distant of the four species, forming a third group (figure 4). The UPGMA tree confirmed this pattern (figure 5). Phylogenetic analyses based on mtDNA identified four distinct clades which differ from current taxonomy (Arbogast *et al.* 2006). *M. macdonaldi*, *M. melanotis* and *M. parvulus* from Genovesa clustered on the same branch despite belonging to three different species and formed the most distant branch within the Galápagos mockingbird genus. All other *M. parvulus* grouped in a second branch except for individuals from Isabela which formed a third phylogenetically divergent class. *M. trifasciatus* formed a fourth branch. Our micorsatellite data confirm this pattern except that individuals from Genovesa did not group with *M. macdonaldi* and *M. melanotis* but instead with the other *M. parvulus* populations (figure 5). Differential introgression of mitochondrial and nuclear genes might be responsible for this pattern (Arbogast *et al.* 2006). Alternatively, the discrepancy between the nuclear and mitochondrial data could arise from the differing lineage sorting times of the two types of markers. Given the recent evolutionary history of the Galápagos bird fauna, contrasting nuclear and mtDNA patterns are not surprising and have indeed been found in other phylogenies of Galápagos birds (Petren *et al.* 1999, 2005; Bollmer *et al.* 2006).

Based on mitochondrial DNA data, Arbogast *et al.* (2006) suggested that Galápagos mockingbirds diverged approximately 1.6–5.5 Mya following a single colonization event, thus forming a monophyletic clade. Our data do not provide information about the colonization since we did not include taxa from outside Galápagos as outgroups in our study. However, we can compare the divergence patterns within Galápagos between the published mtDNA and our microsatellite data. The mtDNA data suggested that *M. macdonaldi*, *M. melanotis* and *M. parvulus* from Genovesa were the first to split from all others (Arbogast *et al.* 2006), while our microsatellite data suggest that *M. trifasciatus* diverged before *M. macdonaldi* and *M. melanotis* (figure 5). Both patterns of diversification generally match information

about island age and the directionality of prevailing winds (Colinvaux 1984). *M. trifasciatus*, *M. macdonaldi* and *M. melanotis* inhabit the eastern-most and oldest islands of the Galápagos archipelago (figure 1 and table 1), likely to be colonized first. Within Galápagos, the phylogenetic pattern detected here is consistent with a model of wind-based dispersal following initial colonization, i.e. from the southeast to the northwest, as previously suggested by Arbogast *et al*. (2006). Further investigations with nuclear genetic markers will hopefully improve our understanding of the relationship between the Galápagos mockingbirds and their continental relatives, their time of divergence and rate of diversification, and resolve the contrasting pattern found between the mitochondrial and nuclear DNA.

(c) *Conclusions*

Using observations on a temporal and spatial scale, we have quantified the effects of small population size and drift on the genetic diversity and structuring at neutral loci of mockingbird populations in Galápagos. Temporally, we measured the change in genetic diversity over 100 years (approx. 25 generations) and spatially we compared many different-sized populations across the archipelago. Although archipelago-wide genetic diversity did not change significantly over the last century, genetic drift was pronounced in small populations where it led to substantial variation in allele frequencies over time and to loss of genetic diversity. This was particularly obvious in the tiny Champion population. The significant isolation-by-distance pattern implies that gene flow occurs but the high levels of interisland differentiation emphasize the existence of substantial barriers to gene flow between islands.

We conclude that for the mockingbird populations in Galápagos, genetic drift is strong and gene flow limited. Our results contrast with the findings in Darwin's finches where interisland migration is widespread, isolation-by-distance is weak and substantial genetic drift has only been found in populations of the warbler finch on small, peripheral islands (Petren *et al*. 2005; Tonnis *et al*. 2005). Future studies will have to show whether, in contrast to the situation in Darwin's finches, isolation and genetic drift may have contributed to the phenotypic divergence among mockingbird populations.

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REFERENCES

Anderson, E. C. 2005 An efficient Monte Carlo method for estimating N-e from temporally spaced samples using a coalescent-based likelihood. *Genetics* **170**, 955–967. (doi:10.1534/genetics.104.038349)

Arbogast, B. S., Drovetski, S. V., Curry, R. L., Boag, P. T., Seutin, G., Grant, P. R., Grant, B. R. & Anderson, D. J. 2006 The origin and diversification of Galapagos mockingbirds. *Evolution* **60**, 370–382.

Barton, N. H. 1998 Natural selection and random genetic drift as causes of evolution on islands. In *Evolution on islands* (ed. P. R. Grant). New York, NY: Oxford University Press.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. 2004 GENETIX 4.05, *logiciel sous Windows TM pour la génétique des populations*. Montpellier, France: Université de Montpellier II.

Bollmer, J. L., Whiteman, N. K., Cannon, M. D., Bednarz, J. C., De Vries, T. & Parker, P. G. 2005 Population genetics of the Galapagos hawk (*Buteo galapagoensis*): genetic monomorphism within isolated populations. *The Auk* **122**, 1–15.

Bollmer, J. L., Kimball, R. T., Whiteman, N. K., Sarasola, J. H. & Parker, P. G. 2006 Phylogeography of the Galapagos hawk (Buteo galapagoensis): a recent arrival to the Galapagos Islands. *Mol. Phylogenet. Evol.* **39**, 237–247. (doi:10.1016/j.ympev.2005.11.014)

Brandstrom, M. & Ellegren, H. 2008 Genome-wide analysis of microsatellite polymorphism in chicken circumventing the ascertainment bias. *Genome Res.* **18**, 881–887. (doi:10.1101/gr.075242.107)

Ciofi, C., Milinkovitch, M. C., Gibbs, J. P., Caccone, A. & Powell, J. R. 2002 Microsatellite analysis of genetic divergence among populations of giant Galapagos tortoises. *Mol. Ecol.* **11**, 2265–2283. (doi:10.1046/j.1365-294X.2002.01617.x)

Colinvaux, P. A. 1984 The Galapagos climate: past and present. In *Galapagos* (*key environments*) (ed. R. Perry). Oxford, UK: Pergamon.

Crow, J. F. & Kimura, M. 1970 *An introduction to population genetic theory*. New York, NY: Harper and Row Publishers.

Curry, R. L. 1986 Whatever happened to the Floreana mockingbird? *Noticias de Galapagos* **43**, 13–15.

Darwin, C. 1839 *Voyage of the beagle*. London, UK: Henry Colburn.

Darwin, C. R. 1859 *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. London, UK: John Murray.

Ellegren, H., Primmer, C. R. & Sheldon, B. C. 1995 Microsatellite evolution—directionality or bias. *Nat. Genet.* **11**, 360–362. (doi:10.1038/ng1295-360)

Frankham, R. 1996 Relationship of genetic variation to population size in wildlife. *Conserv. Biol.* **10**, 1500–1508. (doi:10.1046/j.1523-1739.1996.10061500.x)

Frankham, R. 1997 Do island populations have less genetic variation than mainland populations? *Heredity* **78**, 311–327. (doi:10.1038/hdy.1997.46)

Franklin, I. R. & Frankham, R. 1998 How large must populations be to retain evolutionary potential? *Anim. Conserv.* **1**, 69–70. (doi:10.1111/j.1469-1795.1998.tb00228.x)

Goudet, J. 2001 FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Lausanne, Switzerland: Lausanne University.

Grant, P. R. 1998 *Evolution on islands*. New York, NY: Oxford University Press.

Grant, P. R., Curry, R. L. & Grant, B. R. 2000 A remnant population of the Floreana mockingbird on Champion island, Galapagos. *Biol. Conserv.* **92**, 285–290. (doi:10.1016/S0006-3207(99)00092-0)

Hamilton, T. H. & Rubinoff, I. 1967 On predicting insular variation in endemism and sympatry for Darwin finches in Galapagos Archipelago. *Am. Nat.* **101**, 161.

Harris, M. P. 1974 *The birds of Galapagos*. New York, NY: Houghton-Mifflin.

Hedrick, P. W. 1999 Perspective: highly variable loci and their interpretation in evolution and conservation. *Evolution* **53**, 313–318. (doi:10.2307/2640768)

Hoeck, P. E. A., Beaumont, M. A., James, K. E., Grant, B. R., Grant, P. R. & Keller, L. F. In press *a*. Saving Darwin's muse: evolutionary genetics for the recovery of the Floreana mockingbird. *Biol. Lett*.

Hoeck, P. E. A., Bucher, T. B., Wandeler, P. & Keller, L. F. In press *b*. Microsatellite primers for the four Galápagos mockingbird species (*Mimus parvulus*, *M. macdonaldi*, *M. melanotis*, and *M. trifasciatus*). *Mol. Ecol. Resources*.

Hughes, C. R. & Deloach, D. M. 1997 Developing microsatellites when they are rare: trinucleotide repeat loci in the northern mockingbird Mimus polyglottos. *Mol. Ecol.* **6**, 1099–1102. (doi:10.1046/j.1365-294X.1997.d01-109.x)

Langella, O. 2000 Populations 1.2.30. *Laboratoire Populations, Génétique et Evolution*, Centre National de la Recherche Scientifique, CNRS UPR9034, Gif Sur Yvette, France.

Nei, M. 1972 Genetic distance between populations. *Am. Nat.* **106**, 283.

Paetkau, D., Waits, L. P., Clarkson, P. L., Craighead, L. & Strobeck, C. 1997 An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. *Genetics* **147**, 1943–1957.

Palstra, F. P. & Ruzzante, D. E. 2008 Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? *Mol. Ecol.* **17**, 3428–3447. (doi:10.1111/j.1365-294X.2008.03842.x)

Pertoldi, C., Barker, S. F., Madsen, A. B., Jorgensen, H., Randi, E., Munoz, J., Baagoe, H. J. & Loeschcke, V. 2008 Spatio-temporal population genetics of the Danish pine marten (*Martes martes*). *Biol. J. Linn. Soc.* **93**, 457–464. (doi:10.1111/j.1095-8312.2007.00892.x)

Petren, K., Grant, B. R. & Grant, P. R. 1999 A phylogeny of Darwin's finches based on microsatellite DNA length variation. *Proc. R. Soc. Lond. B* **266**, 321–329. (doi:10.1098/rspb.1999.0641)

Petren, K., Grant, P. R., Grant, B. R. & Keller, L. F. 2005 Comparative landscape genetics and the adaptive radiation of Darwin's finches: the role of peripheral isolation. *Mol. Ecol.* **14**, 2943–2957. (doi:10.1111/j.1365-294X.2005.02632.x)

Prychitko, T. M. & Moore, W. S. 1997 The utility of DNA sequences of an intron from the beta-fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). *Mol. Phylogenet. Evol.* **8**, 193–204. (doi:10.1006/mpev.1997.0420)

Raymond, M. & Rousset, F. 1995 Genepop (v. 1.2)—population-genetics software for exact tests and ecumenicism. *J. Heredity* **86**, 248–249.

Santiago-Alarcon, D., Tanksley, S. M. & Parker, P. G. 2006 Morphological variation and genetic structure of Galapagos Dove (*Zenaida galapagoensis*) populations: issues in conservation for the Galapagos bird fauna. *Wilson J. Ornithol.* **118**, 194–207. (doi:10.1676/05-010.1)

Sefc, K. M., Payne, R. B. & Sorenson, M. D. 2003 Microsatellite amplification from museum feather samples: effects of fragment size and template concentration on genotyping errors. *The Auk* **120**, 982–989. (doi:10.1642/0004-8038(2003)120[0982:MAFMFS]2.0.CO;2)

Slatkin, M. 1985 Gene flow in natural populations. *Annu. Rev. Ecol. Syst.* **16**, 393–430. (doi:10.1146/annurev.ecolsys.16.1.393)

Slatkin, M. 1995 A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**, 457–462.

Takezaki, N. & Nei, M. 1996 Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**, 389–399.

Taylor, S. S., Jamieson, I. G. & Wallis, G. P. 2007 Historic and contemporary levels of genetic variation in two New Zealand passerines with different histories of decline. *J. Evol. Biol.* **20**, 2035–2047. (doi:10.1111/j.1420-9101.2007.01362.x)

Tonnis, B., Grant, P. R., Grant, B. R. & Petren, K. 2005 Habitat selection and ecological speciation in Galapagos warbler finches (*Certhidea olivacea* and *Certhidea fusca*). *Proc. R. Soc. B* **272**, 819–826. (doi:10.1098/rspb.2004.3030)

Tzika, A. C. *et al.* 2008 Population genetics of Galapagos land iguana (genus Conolophus) remnant populations. *Mol. Ecol.* **17**, 4943–4952. (doi:10.1111/j.1365-294X.2008.03967.x)

Valiere, N. 2002 Gimlet: a computer program for analysing genetic individual identification data. *Mol. Ecol. Notes* **2**, 377–379. (doi:10.1046/j.1471-8286.2002.00228.x)

Wandeler, P., Hoeck, P. E. A. & Keller, L. F. 2007 Back to the future: museum specimens in population genetics. *Trends Ecol. Evol.* **22**, 634–642. (doi:10.1016/j.tree.2007.08.017)

Wang, J. L. 2001 A pseudo-likelihood method for estimating effective population size from temporally spaced samples. *Genet. Res.* **78**, 243–257. (doi:10.1017/S0016672301005286)

Weir, B. S. & Cockerham, C. C. 1984 Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370. (doi:10.2307/2408641)

Wright, S. 1943 Isolation by distance. *Genetics* **28**, 114–138.

APPENDIX

Table A: Pair-wise Ds below and F_{ST} above diagonal of all contemporary populations and, separately, all historic populations. Non-significant F_{ST} values are designated with *.

Contemporary populations:	Champion	Espanola, PC	Espanola, MZ	Fernandina	Gardner-by-Espanola	Gardner-by-Floreana	Genovesa	Isabela, AL	Isabela, VL	Marchena	Pinta	Rabida	San Cristobal, S	San Cristobal, N	Santiago	StCruz, GP	StCruz, PtA	StFe
Champion	0	0.829	0.846	0.717	0.892	0.533	0.772	0.683	0.672	0.673	0.757	0.744	0.792	0.812	0.661	0.667	0.710	0.818
Espanola, PC	1.831	0	0.001*	0.524	0.072*	0.731	0.623	0.519	0.526	0.537	0.572	0.606	0.454	0.416	0.514	0.528	0.528	0.643
Espanola, MZ	1.781	0.004	0	0.482	0.054*	0.717	0.601	0.480	0.480	0.499	0.541	0.565	0.413	0.381	0.464	0.475	0.475	0.627
Fernandina	1.042	0.591	0.589	0	0.465	0.565	0.426	0.013	0.024	0.249	0.404	0.317	0.411	0.403	0.277	0.271	0.277	0.465
Gardner-by-Espanola	1.771	0.026	0.023	0.602	0	0.730	0.613	0.466	0.459	0.479	0.533	0.555	0.424	0.398	0.439	0.445	0.445	0.648
Gardner-by-Floreana	0.285	1.877	1.827	0.941	1.988	0	0.647	0.532	0.530	0.540	0.625	0.588	0.649	0.662	0.524	0.510	0.502	0.650
Genovesa	1.163	0.767	0.761	0.524	0.796	1.161	0	0.402	0.400	0.450	0.487	0.417	0.465	0.475	0.365	0.300	0.312	0.524
Isabela, AL	0.935	0.605	0.606	0.024	0.619	0.786	0.467	0	0.033	0.238	0.404	0.321	0.422	0.413	0.272	0.268	0.270	0.456
Isabela, VL	1.030	0.712	0.705	0.037	0.725	0.883	0.527	0.042	0	0.253	0.393	0.304	0.408	0.402	0.261	0.250	0.252	0.440
Marchena	1.089	0.756	0.764	0.326	0.760	0.918	0.669	0.297	0.369	0	0.286	0.275	0.442	0.437	0.174	0.183	0.188	0.432
Pinta	1.237	0.678	0.686	0.622	0.693	1.242	0.620	0.617	0.678	0.347	0	0.236	0.445	0.450	0.143	0.220	0.213	0.464
Rabida	1.136	0.909	0.894	0.448	0.937	1.040	0.475	0.448	0.475	0.371	0.237	0	0.410	0.424	0.131	0.137	0.169	0.425
SanCristobal, S	1.547	0.353	0.344	0.676	0.381	1.502	0.549	0.709	0.783	0.884	0.664	0.640	0	0.021*	0.351	0.332	0.315	0.525
SanCristobal, N	1.553	0.277	0.273	0.612	0.303	1.558	0.540	0.642	0.723	0.816	0.642	0.652	0.028	0	0.352	0.335	0.316	0.523
Santiago	1.037	0.723	0.717	0.467	0.743	0.932	0.474	0.432	0.474	0.240	0.154	0.166	0.635	0.615	0	0.102	0.104	0.338
StCruz, GP	1.075	0.817	0.811	0.494	0.853	0.897	0.339	0.456	0.486	0.275	0.286	0.185	0.623	0.607	0.178	0	0.018*	0.323
StCruz, PtA	1.002	0.694	0.693	0.510	0.733	0.762	0.336	0.461	0.503	0.290	0.266	0.242	0.539	0.516	0.194	0.068	0	0.299
StFe	1.140	0.743	0.752	0.637	0.769	1.049	0.552	0.618	0.666	0.605	0.523	0.481	0.717	0.644	0.417	0.401	0.315	0

Historic populations:	Baltra-CAS	Champion-CAS	Espanola-CAS	Gardner-by-Espanola-CAS	Gardner-by-Floreana-CAS	Genovesa-CAS	Isabela-CAS	Marchena-CAS	Rabida-CAS	San Cristobal-CAS	Santiago-CAS	StCruz-CAS	StFe-CAS
Baltra-CAS	0	0.508*	0.413	0.446*	0.467*	0.390	0.255	0.179	0.219	0.342	0.104	0.024*	0.363*
Champion-CAS	0.928	0	0.738*	0.813*	0.469	0.748*	0.532*	0.577	0.601	0.642	0.508	0.477	0.752*
Espanola-CAS	0.640	1.618	0	0.084*	0.682	0.632	0.416	0.439	0.542	0.326	0.380	0.398	0.602*
Gardner-by-Espanola-CAS	0.708	1.496	0.041	0	0.721	0.692	0.445	0.471	0.583	0.378	0.415	0.429	0.669*
Gardner-by-Floreana-CAS	0.840	0.308	1.665	1.722	0	0.673	0.488	0.532	0.545	0.613	0.478	0.435	0.658
Genovesa-CAS	0.414	1.231	0.821	0.896	1.125	0	0.441	0.485	0.476	0.530	0.401	0.359	0.628*
Isabela-CAS	0.470	0.959	0.579	0.620	0.857	0.527	0	0.243	0.294	0.405	0.243	0.235	0.455*
Marchena-CAS	0.288	1.171	0.627	0.671	1.086	0.635	0.361	0	0.333	0.409	0.192	0.174	0.491*
Rabida-CAS	0.303	1.151	0.964	1.115	0.987	0.529	0.416	0.508	0	0.458	0.196	0.177	0.519*
SanCristobal-CAS	0.593	1.497	0.290	0.333	1.568	0.677	0.743	0.731	0.831	0	0.331	0.335	0.539*
Santiago-CAS	0.176	1.030	0.546	0.624	0.962	0.485	0.404	0.289	0.252	0.544	0	0.074	0.365*
StCruz-CAS	0.072	0.972	0.683	0.778	0.815	0.414	0.428	0.284	0.239	0.625	0.124	0	0.319*
StFe-CAS	0.389	1.109	0.714	0.745	1.006	0.652	0.604	0.686	0.691	0.754	0.428	0.358	0

Table B: Effective population sizes (harmonic mean Ne) with best estimator and lower and upper support limits, and temporal F_{ST} values for the 12 populations for which we had historic and contemporary samples. Non-significant F_{ST} values are designated with *.

	Best Ne	Lower Support Limit	Upper Support Limit	Temporal F_{ST}
Champion	43.39	17.29	107.31	0.277
Espanola	424.69	217.33	1049.65	0.024*
Gardner-by-Espanola	115.61	47.54	496.37	0.106
Gardner-by-Floreana	132.74	74.92	244.64	0.076
Genovesa	745.96	268.17	-	0.027*
Isabela	1590.86	744.44	10108.19	0.021
Marchena	808.69	330.87	-	0.016*
Rabida	238.76	135.65	457.58	0.047
SanCristobal	1206.46	406.75	-	0.017*
Santiago	1363.4	551.03	-	0.006*
StCruz	1116.88	474.97	-	0.009*
StFe	513.17	169.58	-	0.012*

SAVING DARWIN'S MUSE: EVOLUTIONARY GENETICS FOR THE RECOVERY OF THE FLOREANA MOCKINGBIRD

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Saving Darwin's muse: evolutionary genetics for the recovery of the Floreana mockingbird

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The distribution of mockingbird species among the Galápagos Islands prompted Charles Darwin to question, for the first time in writing, the 'stability of species'. Some 50 years after Darwin's visit, however, the endemic Floreana mockingbird (*Mimus trifasciatus*) had become extinct on Floreana Island and, today, only two small populations survive on two satellite islets. As Darwin noted, rarity often precedes extinction. To avert extinction, plans are being developed to reintroduce *M. trifasciatus* to Floreana. Here, we integrate evolutionary thinking and conservation practice using coalescent analyses and genetic data from contemporary and museum samples, including two collected by Darwin and Robert Fitzroy on Floreana in 1835. Our microsatellite results show substantial differentiation between the two extant populations, but our coalescence-based modelling does not indicate long, independent evolutionary histories. One of the populations is highly inbred, but both harbour unique alleles present on Floreana in 1835, suggesting that birds from both islets should be used to establish a single, mixed population on Floreana. Thus, Darwin's mockingbird specimens not only revealed to him a level of variation that suggested speciation following geographical isolation but also, more than 170 years later, return important information to their place of origin for the conservation of their conspecifics.

Keywords: museum specimens; genetic diversity; conservation; Galápagos; Nesomimus

> Rarity, as geology tells us, is the precursor to extinction. We can, also, see that any form represented by few individuals will, during fluctuations in the seasons or in the number of its enemies, run a good chance of utter extinction.
>
> (Darwin 1859, p. 109)

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1. INTRODUCTION

When Charles Darwin visited Galápagos in 1835, he observed and collected specimens of three different mockingbird species (Gould 1837). Darwin noticed the Floreana mockingbird's evident difference from the specimens he collected on other islands. Thus, the former holds a special claim on being a first vital clue for Darwin's theory of speciation under natural selection (Darwin 1839; Steinheimer 2004). The Floreana mockingbird became extinct on Floreana some 50 years after Darwin's visit, most likely owing to introduced predators such as black rats (Curry 1986) and other habitat alterations following human colonization (Charles Darwin Foundation 2008). Today, this endemic species is critically endangered and only occurs on Floreana's satellite islets Champion (20–53 individuals; Grant *et al.* 2000) and Gardner-by-Floreana (200–500 birds; P. E. A. Hoeck & L. F. Keller 2009, unpublished census data; figure 1).

As Darwin (1859) noted, rarity often precedes extinction. To avert extinction, a recovery plan has been drafted to restore natural habitat on Floreana (Charles Darwin Foundation 2009) and to reintroduce *Mimus trifasciatus* (Charles Darwin Foundation 2008). Owing to isolation since the extinction of the geographically interjacent Floreana population, Grant *et al.* (2000) predicted genetic differentiation and loss of genetic variation in the two remnant populations (figure 1). Therefore, a key question raised by the recovery plan is whether the two satellite populations have been evolving independently since well before the human-induced decline of the Floreana population.

Here we show that coalescent-based models combined with genetic data from contemporary samples and museum specimens—including two collected by Darwin and Fitzroy on Floreana—can be used to estimate divergence times and inform the reintroduction strategy.

2. MATERIAL AND METHODS

(a) *Sample collection*

We obtained blood samples from the Champion and Gardner populations in 2006–2008 ('2008 samples'; figure 1) and small toe-pad samples from specimens collected on both satellite islets in 1905/1906 ('1906 samples'; figure 1) by a California Academy of Sciences (CAS) expedition. We also analysed two specimens from Floreana itself collected in 1835 ('Floreana specimens'; figure 1) and held at the Natural History Museum (NHM, London, UK).

(b) *Genotyping*

Seventeen microsatellite loci were amplified from all samples as described elsewhere (Hoeck *et al.* 2009), 11 of which displayed polymorphism. Contemporary samples were genotyped once, except in the case of amplification failure, in which case PCR was repeated. CAS historic samples were genotyped four times at each locus (Hoeck *et al.* in press). Genotyping of the two Floreana specimens was verified by repeating the fragment analysis eight to 16 times per sample and by control amplifications at the NHM. Genotyping error rates per locus and consensus genotypes were determined using GIMLET (Valiere 2002) and results crosschecked by hand. Genotyping details and success rates are described in the electronic supplementary material.

(c) *Genetic diversity and differentiation*

Genotypic and Hardy–Weinberg equilibrium were tested as described in the electronic supplementary material. Genetic diversity within populations was measured as number of alleles, allelic richness, expected heterozygosity and number of private alleles. Differentiation between contemporary and historic populations was estimated using Nei's D_s (Langella 2000) and F_{ST} (Goudet 2001). An analysis of molecular variance (AMOVA) was conducted using ARLEQUIN v. 3.11 (Excoffier *et al.* 2005).

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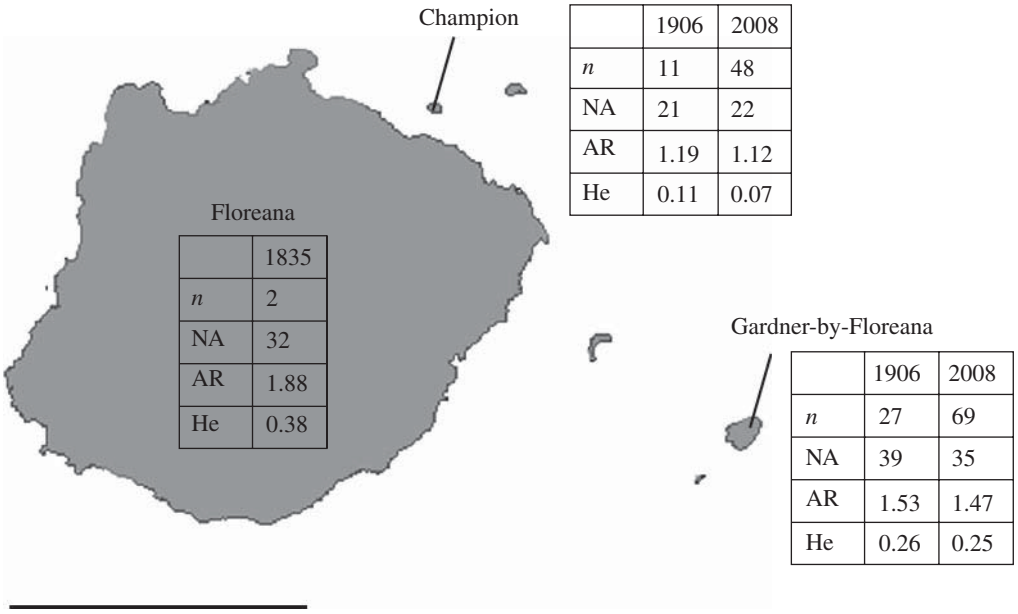

	1906	2008
n	11	48
NA	21	22
AR	1.19	1.12
He	0.11	0.07

	1835
n	2
NA	32
AR	1.88
He	0.38

	1906	2008
n	27	69
NA	39	35
AR	1.53	1.47
He	0.26	0.25

Figure 1. Floreana (17 253 ha) and its satellite islets Champion (9.4 ha, less than 1 km away) and Gardner (81 ha, approx. 8 km from Floreana). Number of samples (*n*) obtained from each island and time period and estimates of genetic diversity (NA, number of alleles; AR, allelic richness; He, expected heterozygosity). Scale bar, 10 km.

(**d**) ***Coalescent modelling***

The genetic data were analysed in a Bayesian framework using a coalescent-based model. No methods currently exist that can incorporate historic and current samples in a model with divergent populations. Here, based on the model of Beaumont (2003), we developed a new approach that can, in principle, accommodate any number of populations and branching topologies (see the electronic supplementary material for details). We modelled populations diverging from a common ancestral population in the framework introduced by Beaumont (2003) to compute Bayesian posterior distributions for demographic parameters using temporally sampled genetic data (figure 2*a*; Beaumont 2003). We performed the analysis with two sets of priors (see the electronic supplementary material for details). One analysis was performed with narrower, gamma-distributed, priors on Ne, based on census information from Champion and Gardner (Grant *et al.* 2000; P. E. A. Hoeck & L. F. Keller 2009, unpublished census data), and the other analysis assumed that the prior on Ne was uniform between 0 and 10 000 for all islands. The priors used for generation time and times of divergence were the same for either analysis, and were uniform distributions with upper and lower bounds based on reasoned guesses as to minimum and maximum possible values (electronic supplementary material). The commented C code for the model is available at http://www.rubic.reading.ac.uk/~mab/stuff/mocking_analysis.zip.

We compared two scenarios of divergence: one in which the Gardner population first started to diverge from the common ancestral population, followed by divergence between Floreana and Champion (topology 1, figure 2*a*), and another in which Champion first diverged (topology 2). We used genetic data from a total of six sampling occasions for our analyses (electronic supplementary material).

3. RESULTS AND DISCUSSION

The contemporary Champion and Gardner populations are clearly divergent (pairwise $F_{ST} = 0.533$, $p < 0.005$; $D_s = 0.26$) owing, in part, to the low genetic diversity observed on Champion and pronounced genetic drift over the last century (figure 1). Between 1906 and 2008 Champion lost 39 per cent of its expected heterozygosity, as predicted theoretically by Grant *et al.* (2000) based on observed population sizes, whereas Gardner lost only 6 per cent (figure 1). The extent of genetic drift over the last century is exemplified by the fact that an allele with a frequency as high as 0.64 in the Champion population in 1906 was completely lost by 2008. Although genetic drift since 1906 contributed somewhat to the divergence of the two satellite populations in 2008 (pairwise F_{ST} increased by 0.06 from 1906 to 2008), the two populations were already considerably differentiated in 1906 ($F_{ST} = 0.47$, $p < 0.005$; $D_s = 0.23$). This finding was corroborated by an AMOVA, in which 46 per cent of the genetic variation was found among islands, 6 per cent between the two time periods within Champion and Gardner, respectively, and 48 per cent within time period and population (*p*-values for all comparisons less than 0.001).

Is the divergence in 1906 evidence that the two satellite populations have been evolving independently since long before the human-induced decline of the interjacent population on Floreana, or does it reflect an evolutionary young phenomenon related to that decline? Our coalescent analyses suggest that the Gardner population started to diverge from the Floreana population before the Champion population did (the posterior probability of topology 1 was 0.83). This is not surprising given the closer geographical proximity of Champion and Floreana (figure 1) and the south–southeast direction of prevailing winds that could act as a deterrent to immigration from Floreana to Gardner. The estimates of divergence times were well bounded and suggest a relatively recent divergence (figure 2*b*,*c*). Under topology 1 (Gardner diverged first; figure 2*a*), we estimated a mode of approximately 270 years ago for the younger divergence of Champion (figure 2*b*) and a mode of 450 years ago for the older divergence of Gardner (figure 2*c*). Results under topology 2 (Champion diverged first) differed somewhat, but not fundamentally (figure 2*b*,*c*). Under any scenario, it is highly unlikely that any divergence began more than 800 years ago (figure 2*c*).

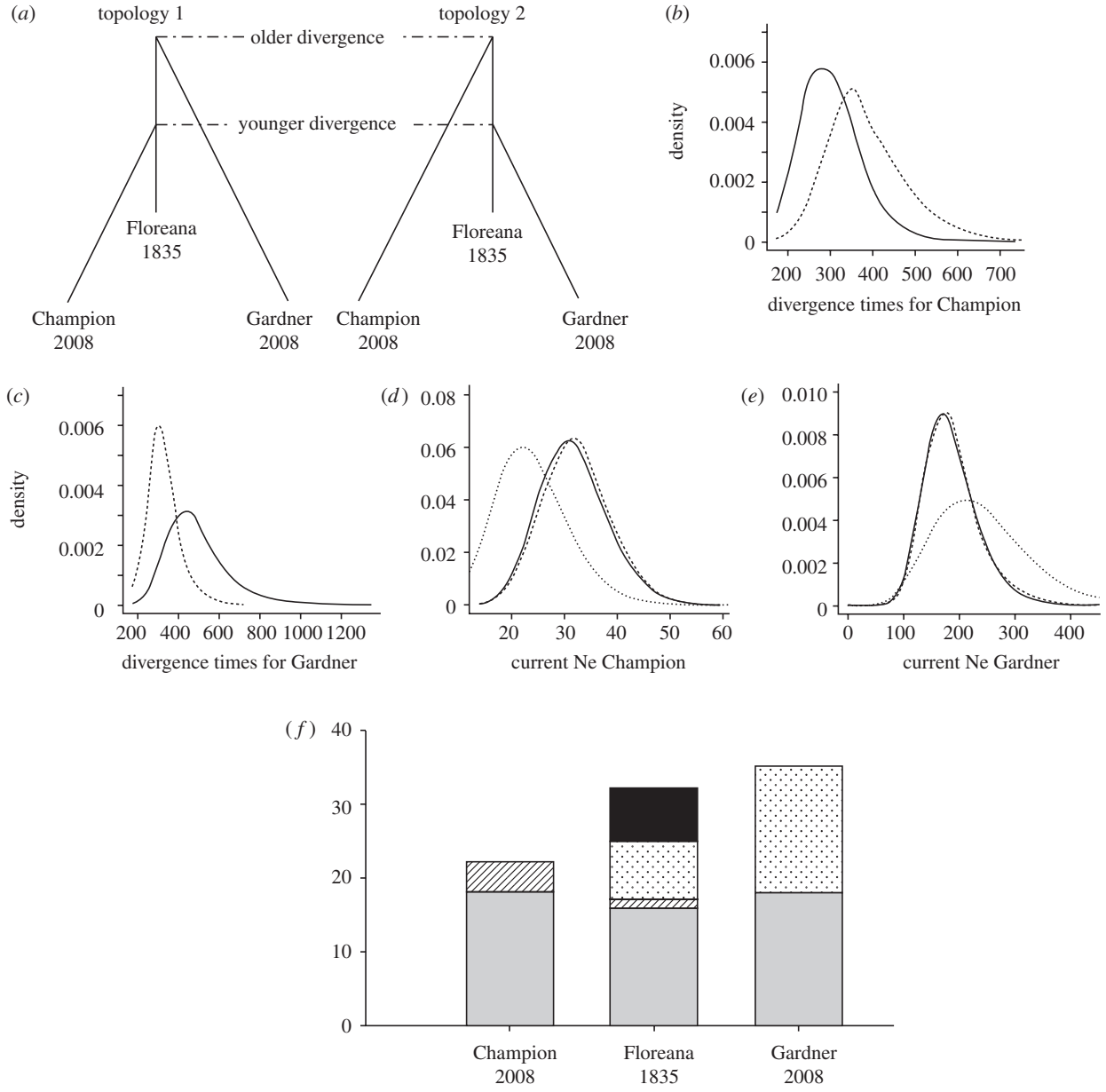

Figure 2. (*a*) The two topologies of population divergence investigated with the coalescent models with the Gardner (topology 1) or the Champion (topology 2) population diverging first. (*b*,*c*) Posterior probability distributions of the divergence times for Champion and Gardner under the two topologies. Solid lines, topology 1; dashed lines, topology 2. (*d*,*e*) Posterior probability distribution of the current effective population size (Ne) on Champion and Gardner, respectively. Current Ne values refer to the harmonic mean Ne between 1906 and 2008. The dotted lines represent the informative prior, based on the information from the census population sizes. Solid lines, topology 1; dashed lines, topology 2. (*f*) Total number of alleles across all loci in the contemporary Champion and Gardner populations, and in the two Floreana specimens. Grey: alleles that occurred in both contemporary populations and in the Floreana specimens. Alleles today only found on Gardner (dotted) or Champion (hatched), or only detected in the Floreana specimens (black).

Our coalescent analyses, therefore, do not support the hypothesis that this divergence is ancient. Rather, they are compatible with a model of three somewhat subdivided populations connected by recurrent gene flow. With the decline and ultimate extinction of the Floreana population, gene flow declined, as indicated by the private alleles, some of them with frequencies as high as 1, in both Gardner and Champion in 2008 (figure 2*f*). A former connection through gene flow is further supported by the finding that the two Floreana specimens shared 16 (50%) of their alleles with both contemporary populations, but also harboured alleles that today are private to either one of the satellite populations (figure 2*f*). Thus, part of the variation once present on Floreana has persisted in both satellite populations. Our results therefore suggest that current and future management actions should focus on conserving the two satellite populations *in situ* and establishing a single third population on Floreana using birds from both islets to maximize genetic diversity upon which selection can act. This view is further supported by evidence from another avian radiation in Galápagos, Darwin's finches, where interbreeding of species has increased the additive genetic variance in

morphological traits under selection, which may facilitate evolution along novel trajectories (Grant & Grant 2008). Nothing could better prepare a reintroduced population for the future. The short divergence times between the Gardner and Champion population do not imply that no evolutionary changes have occurred or that individuals are not locally adapted. Instead, they imply that mixing of the two populations will probably lead to heterosis in addition to increased additive genetic variance. Theory-based research suggests that this is likely when divergence levels are relatively high and effective population sizes relatively low (Whitlock *et al*. 2000) as is the case in the Floreana mockingbird (figure 2*d*,*e*).

All fieldwork and procedures were approved by the Galápagos National Park Service.

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Beaumont, M. A. 2003 Estimation of population growth or decline in genetically monitored populations. *Genetics* **164**, 1139–1160.

Charles Darwin Foundation 2008 *The reintroduction of the Floreana mockingbird to its island of origin*. Puerto Ayora, Galapagos: Charles Darwin Foundation and Galapagos National Park Service.

Charles Darwin Foundation 2009 *Project Floreana 2009–2013: an integrated approach*. Puerto Ayora, Galapagos: Charles Darwin Foundation.

Curry, R. L. 1986 Whatever happened to the Floreana mockingbird? *Noticias de Galapagos* **43**, 13–15.

Darwin, C. R. 1839 *Narrative of the surveying voyages of His Majesty's Ships Adventure and Beagle between the years 1826 and 1836, describing their examination of the southern shores of South America, and the Beagle's circumnavigation of the globe. Journal and remarks 1832–1836*. London, UK: Henry Colburn.

Darwin, C. R. 1859 *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. London, UK: John Murray.

Excoffier, L., Laval, G. & Schneider, S. 2005 Arlequin v. 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinform. Online* **1**, 47–50.

Goudet, J. 2001 *FSTAT, a program to estimate and test gene diversities and fixation indices* (*version 2.9.3*). Lausanne, Switzerland: Lausanne University.

Gould, J. 1837 Three species of the genus *Orpheus*, from the Galapagos, in the collection of Mr Darwin. *Proc. Zool. Soc. Lond.* **5**, 27.

Grant, P. R. & Grant, B. R. 2008 *How and why species multiply. The radiation of Darwin's finches*. Princeton, NJ: Princeton University Press.

Grant, P. R., Curry, R. L. & Grant, B. R. 2000 A remnant population of the Floreana mockingbird on Champion island, Galapagos. *Biol. Conserv.* **92**, 285–290. (doi:10.1016/S0006-3207(99)00092-0)

Hoeck, P. E. A., Bollmer, J. L., Parker, P. G. & Keller, L. F. In press. Differentiation with drift: a spatio-temporal genetic analysis of Galapagos mockingbird populations (*Mimus* spp.). *Phil. Trans. R. Soc. B*.

Hoeck, P. E. A., Bucher, T. B., Wandeler, P. & Keller, L. F. 2009 Microsatellite primers for the four Galápagos mockingbird species (*Mimus parvulus*, *M. macdonaldi*, *M. melanotis*, and *M. trifasciatus*). *Mol. Ecol. Resour.* **9**, 1538–1541.

Langella, O. 2000 Populations 1.2.30. Laboratoire Populations, Génétique et Evolution. Centre National de la Recherche Scientifique, CNRS. UPR9034, Gif Sur Yvette. See http://bioinformatics.org/~tryphon/populations/.

Steinheimer, F. D. 2004 Charles Darwin's bird collection and ornithological knowledge during the voyage of H.M.S. 'Beagle', 1831–1836. *J. Ornithol.* **145**, 300–320. (doi:10.1007/s10336-004-0043-8)

Valiere, N. 2002 Gimlet: a computer program for analysing genetic individual identification data. *Mol. Ecol. Notes* **2**, 377–379. (doi:10.1046/j.1471-8286.2002.00228.x)

Whitlock, M. C., Ingvarsson, P. K. & Hatfield, T. 2000 Local drift load and the heterosis of interconnected populations. *Heredity* **84**, 452–457. (doi:10.1046/j.1365-2540.2000.00693.x)

DATA SUPPLEMENTS

a) Darwin and the Floreana mockingbirds

When Charles Darwin visited Galápagos aboard HMS Beagle in 1835, he observed and collected specimens of what later turned out to be three different mockingbird species (Gould, 1837, Darwin, 1839a), first from San Cristóbal, then from Floreana, and finally from Isabela and Santiago islands (Darwin, 1839b). He noticed the Floreana mockingbird's difference from the other three Galápagos mockingbird specimens (Darwin, 1839a): "In the case of the mocking-bird, I ascertained (and have brought home the specimens) that one species (*Orpheus trifasciatus*, Gould) is exclusively found in Charles Island [now called Floreana]; a second (*O. parvulus*) on Albemarle Island [now Isabela]; and a third (*O. melanotis*) common to James [now Santiago] and Chatham [now San Cristóbal] Islands. The two last species are closely allied, but the first would be considered by every naturalist as distinct." Today, four endemic, allopatrically living mockingbird species are recognized in the Galápagos (*Mimus macdonaldi, M. melanotis, M. parvulus* and *M. trifasciatus*; Arbogast *et al.*, 2006). Darwin, following Gould, classified the specimen he collected on Santiago as *Orpheus melanotis*. However, mockingbirds from Santiago today are recognized as belonging to the same species as mockingbirds from Isabela, *M. parvulus*, although they are considered members of two different subspecies (Swarth, 1931).

b) Sample collection and microsatellite analysis

Blood of living mockingbirds from Champion and Gardner was collected on filter paper after a small puncture of the wing vein (Hoeck *et al.*, submitted) under permits issued by the Galápagos National Park Service.

Extraction and PCR were performed using previously published methods (Hoeck et al., 2009, Hoeck et al., submitted) and all necessary precautions for work with historic samples were taken (Hoeck et al., submitted, Wandeler et al., 2007). As a further control, duplicate toe pad samples of each of the two Floreana specimens (BMNH 1855.12.19.225, coll. Darwin, holotype, *Orpheus trifasciatus* Gould, 1837; BMNH

1837.2.21.401, coll. Fitzroy) were independently extracted in the Botany laboratory at the Natural History Museum, London.

The following 17 microsatellite loci were amplified for all samples: MpAAT26, Nes01, Nes03, Nes04, Nes06, Nes10, Nes12, Nes13, Nes14, Nes15, Nes16, Nes17, Nes18, Nes19, Nes20, Nes22 and Nes23 (Hoeck et al., 2009, Hoeck et al., submitted).

c) Genetic diversity estimates and genetic differentiation

Genotypic equilibrium between all pairs of loci in each population was tested using G-statistics with Bonferroni corrections (13,600 permutations; FSTAT v2.9.3.2; Goudet, 2001). Deviations from Hardy-Weinberg equilibrium for each locus were assessed with allele randomizations within samples (1,000 permutations per test) and overall samples (10,000 permutations). Genetic diversity within populations was measured as number of alleles (NA), allelic richness (AR) and expected (He) with FSTAT. Number of private alleles was assessed with GENALEX v6 (Peakall and Smouse, 2006).

Differentiation between contemporary and historic populations was estimated using Nei's standard genetic distance D_s (Nei, 1972) as calculated in Populations v1.2.30 (Langella, 2000) and, for comparison, Weir and Cockerham's (Weir and Cockerham, 1984) estimator θ for Wright's F_{ST} (FSTAT). An analysis of molecular variance (AMOVA) was conducted on allele frequencies to test for among and within-population variation using Arlequin v3.11 (Excoffier et al., 2005) and 16,000 permutations.

d) Genotyping results

Quantitative PCR analysis of the Floreana specimen DNA revealed an average concentration of 17 pg/µl and 43 pg/µl respectively and, over all PCR replications, amplification success for the Floreana specimens at the 17 loci was 78%, ranging from 60-97%, with a minimum of 4 successful amplifications per marker and individual. Hence, we were able to successfully genotype 100% of the loci. Combined allelic dropout of all replicates was 20% (SD=0.008) and in one single case (0.5%, SD=0.007) a false allele was detected. DNA extracted and amplified at the NHM as a control revealed the same scoring results as DNA extracted and amplified in Zurich. CAS sample

concentrations ranged from 0.04 to 182 pg/µl (mean: 18 pg/µl± 5.9). Two CAS samples from Champion and 2 from Gardner were excluded from the analyses because less than 50% of the loci amplified successfully in these samples. On average, 80% of the PCR reactions for the CAS specimens resulted in successful amplification (across individuals and loci, SD=0.18), average dropout rate was 6% (SD=0.09) and false allele rate 2.7% (SD=0.03). We were able to successfully genotype 92% of the loci in the CAS specimens and 100% in all of the contemporary samples. No deviations from genotypic or Hardy-Weinberg equilibrium were detected for any loci in any population. Six loci (Nes04, Nes06, Nes10, Nes19, Nes20 and Nes23) did not show any polymorphism in any of the five populations, whereas we identified between 2 and 7 alleles for the polymorphic loci.

e) Coalescent modeling

The data were analysed in a Bayesian framework using a coalescent-based model. A number of likelihood-based packages are potentially available (Hey and Nielsen, 2004), but no methods currently exist that can incorporate historic and current genetic data in a full-likelihood framework in a model with divergent populations. It is possible to use methods based on approximate Bayesian computation, but these necessarily involve some loss of power. Therefore, we developed a full-likelihood method for the specific scenario in the present article based on the model of Beaumont (2003) for temporally sampled genetic data (itself an extension of the method of Berthier *et al.* (2002)).

Specifically, the model of Beaumont (2003) consists of an arbitrarily long temporal series of observations of gene frequencies at a locus, a_0, …, a_d, where a_0 is a vector of gene frequencies (counts, not relative frequencies) at the most recent time and a_d is a vector of gene frequencies at the most distant time. In this model it is assumed that the sampling period is short enough that mutations arising in this period will contribute a negligible amount to gene frequencies. The probability of the frequency distribution a_i can be computed in terms of a hidden variable, f_{i+1}, the gene frequency among ancestral lineages at the time the a_{i+1} sample was taken. Simplifying the formula 5 in Beaumont (2003), we can write

$$P(a_0 \mathrm{K}\ a_d \mid T_{1,\mathrm{K}\,d}) = \int_x \sum_f p(a_0 \mid f_1, T_1)\left[\prod_{i=1}^{d-1} P(a_i, f_i \mid f_{i+1}, T_{i+1})\right] P(a_d, f_d \mid x) P(x) dx$$

where x is the population frequency at the time the dth (earliest) sample is taken and T_i is the duration measured in generations of the time interval between the ith and (i-1)th sample divided by the harmonic mean effective size (*Ne*). Note that there are some notational changes relative to Beaumont (2003). The summation is over all possible gene-frequencies among ancestral lineages, and the integral is over all baseline frequencies given some prior (in all cases here taken to be a flat Dirichlet). In this model, based on coalescent theory, the likelihood depends only on harmonic mean effective size over the interval (Beaumont, 2003), and is invariant to the details of population size change within the interval. A method for computing the likelihood using importance sampling within this framework is described in Beaumont (2003, and see also Anderson, 2005). This estimate of the likelihood can then be plugged into a Metropolis-Hastings sampler, which will converge in expectation onto the true posterior distribution, as is also described in Beaumont (2003) and Andrieu & Roberts (2009).

It is straightforward to apply this model to branching populations by replacing the term $P(a_d, f_d \mid x)$ above with, for example, $P(a_{(1)d}, f_{(1)d} \mid f_{(1)A}, T_{(1)A}) P(f_{(1)A}, f_{(2)A} \mid x)$, which can then be used to 'glue' in any number of populations. Here the subscripts (1) and (2) index populations 1 and 2, and A denotes ancestral. That is, the probability of obtaining observation frequency a_d and (hidden) lineage frequency f_d in population 1 can be computed given the coalescent-scaled time interval $T_{(1)A}$ and the ancestral lineage frequency at the time of the split $f_{(1)A}$, and, for a single branching event, the probability of this latter can be computed jointly with the ancestral frequency of the other population, given the baseline frequency x. By recursively replacing $P(f_{(1)A}, f_{(2)A} \mid x)$ with equivalent expressions for additional populations, any number of populations and branching topologies can be accommodated (see also O'Ryan et al., 1998).

The commented C code for the model is available at:
www.rubic.reading.ac.uk/~mab/stuff/mocking_analysis.zip.

In the present scheme we consider the two scenarios illustrated in Figure S1: From each of the contemporary populations, two samples have been taken roughly one

hundred years apart. For ease of modeling we take these to be 2008 and 1906, respectively, for both Champion and Gardner. An additional recent sample (n=22) was obtained in 1988/89 from Champion. This latter sample was only included in the coalescent models and not in the other analyses because of the short time span to the 2006/8 samples and because analogous samples from Gardner were missing. From Floreana we have the 19th Century sample, which we take to be 1835. In the model we infer 'current' *Ne* for each of the 3 populations. This is defined to be the harmonic mean *Ne* from the present time back to the 1906 sample in the case of the two extant populations, and to the harmonic mean *Ne* back to the younger divergence time for Floreana (as shown in the Fig. S1). The 'ancient' *Ne* is then the harmonic mean effective size from the earlier sample (or split time in the case of Floreana) back to the relevant population split (Fig. S1). In this model, there are 4 further parameters that are inferred: the generation time, the two divergence times (younger divergence: T1, and older divergence: T2), and the topology (topology 1 or 2).

In a Bayesian analysis the aim is to compute the probability distribution of parameters in the model (e.g. the divergence times or the effective population sizes), conditional on the observed data. This is known as the posterior distribution. In order to do so it is necessary to specify an initial probability distribution for the parameters in the model. This is the prior distribution (for simplicity often called the 'prior'), and expresses one's knowledge about the quantity of interest without taking the data into account. The posterior is then given by the prior multiplied by the likelihood function (specifying the probability of the data, given the parameter values, c.f. the formula above), standardized so that the probabilities sum to one. The prior, along with the model itself and its associated assumptions, is chosen subjectively for the problem in hand. In order to examine the sensitivity of the results to the choice of prior we used two models with different sets of prior assumptions. Both models assumed equal prior probabilities for topology 1 and 2, uniform priors between 173 years (corresponding to the year 1835) and 173+1000 for the younger divergence time and uniform priors U(T1,T1+1000) for the older divergence time. The generation time had a uniform prior U(2,4).

Model 1 and Model 2 differed in that Model 1 used informative priors for *Ne* on Champion and Gardner based on a gamma distribution. Specifically, for both ancient and

current *Ne* on Champion we assumed a gamma distribution with shape parameter 12 and scale parameter 2. This gives most support to *Ne* between 10 and 50 with a mean of 24, reflecting the information we have from census data (20-53 individuals; Grant et al., 2000; and PEAH and LFK, unpublished census data) and the expectation that *Ne* is typically smaller than census sizes. Similarly, we assumed a gamma distribution with shape parameter 8.0 and scale parameter 30 for both ancient and current *Ne* on Gardner. This gives most support to *Ne* between 50 and 600, with a mean of 240, again reflecting information from census data (200-500 birds, PEAH and LFK, unpublished census data). However, the accuracy of the census data is much lower on Gardner than on Champion. Thus, we used a wider range for the prior of *Ne* on Gardner. For Floreana we used a uniform prior with bounds to U(0,2000). Model 2 was identical to Model 1, except that is was based on broad, uniform flat priors U(0,10000) on all the *Ne*.

For Model 1, which is our favoured model, with the most informative priors on *Ne*, we find that the posterior probability of topology 1 (Champion branches most recently) is estimated to be 0.83. We find in our model that longer generation times are supported. We see that the posterior distributions for *Ne* in Gardner and Champion tend not to show sharp departures from the prior, although there is a shift towards higher values for the Champion recent *Ne*, and there is no strong dependence on topology. By contrast for ancient *Ne* in Champion the results show a bigger difference with topology, with the topology 1 favouring smaller values. In the case of Gardner the recent *Ne*s are estimated to be somewhat smaller than favoured by the prior, and again insensitive to topology. The ancient *Ne*s for Gardner, like Champion, are closer to the prior but more sensitive to topology, with topology 2 favouring smaller *Ne*. These results are quite straightforward to explain by reference to the joint posterior distributions of *Ne* with divergence time. It can be seen that longer divergence times make large value of *Ne* more probable. In topology 1, with a shorter divergence time for Champion, smaller *Ne* is more probable and vice versa for Gardner. There is little information on *Ne* in Floreana, with large values being favoured, up to the limit set by the prior.

A key observation is that in Model 1, with informative priors, the estimates of divergence times between Champion and Floreana, and between Gardner and Floreana, are well bounded and relatively recent. We estimate a mode of around 100 and 120 years

prior to 1835 for the most recent divergence time for Champion (topology 1) and Gardner (topology 2) respectively, and a mode of 180 and 280 years prior to 1835 for the more ancient divergence time for Champion (topology 2) and Gardner (topology 1). The deepest divergence time is unlikely to be longer than 600 years prior to 1835, and for the most recent divergence time, values of 173 years (i.e. a divergence at the time when the initial samples were taken) have some support for both Champion and Gardner.

These results for the divergence times are strongly dependent on the priors that we have chosen for *Ne*, as can be seen in the results for Model 2. Here, the posterior probability of topology 1 is now 0.68. Looking at the joint distribution of divergence time and *Ne*, we can see that having a relatively uninformative prior on ancestral *Ne* we have no strong power of estimation, in contrast with the estimates of recent *Ne*, which, based on two sampling times, are well estimated. Thus we can have longer divergence times and larger *Ne*, or vice versa. By imposing priors, based on arguments of the densities that the islands can realistically support, we can see that the level of divergence observed, with the values of *Ne* that are possible, can only occur if the divergence times are short. This is compatible with models of recurrent colonisation or immigration to the islands, and certainly does not support a model of ancient divergence. It is important to note that the priors on *Ne* that were imposed were based on arguments concerning the census size, yet these priors are reasonably compatible with the posterior distributions of recent *Ne* for Champion and Gardner, for which we can obtain reasonable estimates. A further point to note is that the different priors used in Model 1 and Model 2 yield different estimates for the generation time, with the mode in Model 1 being four, and the mode for Model 2 being two.

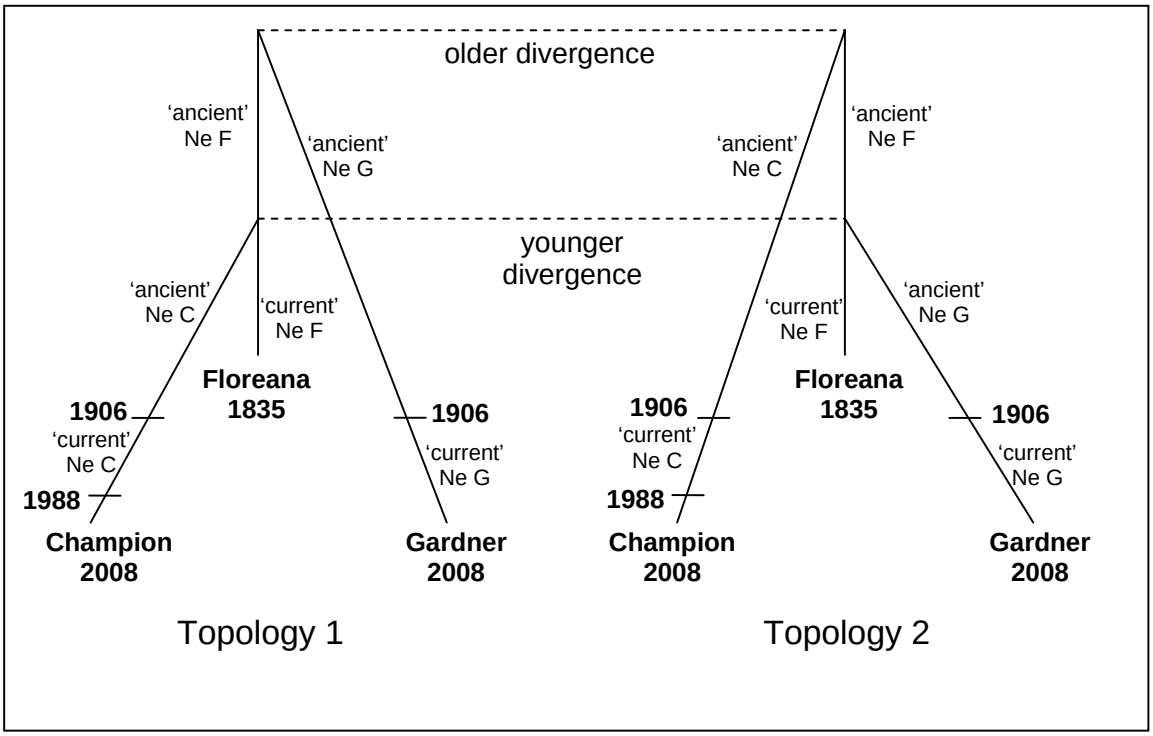

Figure S1. Two scenarios for coalescent models showing the two topologies of population divergence investigated and sampling times. The abbreviations F, C, and G refer to Floreana, Champion, and Gardner, respectively. 'Current *Ne*' refers to the harmonic mean effective population size from 1906 to 2008 in the case of Champion and Gardner. For Floreana 'current' refers to the period from the younger divergence to 1835. For Champion and Gardner 'Ancient *Ne*' is the harmonic mean *Ne* from the time of divergence from Floreana to 1906, and for Floreana 'ancient' refers to the period between the older divergence and the younger divergence.

REFERENCES

ANDERSON, E. C. (2005) An efficient Monte Carlo method for estimating N-e from temporally spaced samples using a coalescent-based likelihood. *Genetics,* 170**,** 955-967.

ANDRIEU, C. & ROBERTS, G. O. (2009) The pseudo-marginal approach for efficient Monte Carlo computations *Annals of Statistics,* 37**,** 697-725.

ARBOGAST, B. S., DROVETSKI, S. V., CURRY, R. L., BOAG, P. T., SEUTIN, G., GRANT, P. R., GRANT, B. R. & ANDERSON, D. J. (2006) The origin and diversification of Galapagos mockingbirds. *Evolution,* 60**,** 370-382.

BEAUMONT, M. A. (2003) Estimation of population growth or decline in genetically monitored populations. *Genetics,* 164**,** 1139-1160.

BERTHIER, P., BEAUMONT, M. A., CORNUET, J. M. & LUIKART, G. (2002) Likelihood-based estimation of the effective population size using temporal changes in allele frequencies: A genealogical approach. *Genetics,* 160**,** 741-751.

DARWIN, C. R. (1839a) *Narrative of the surveying voyages of His Majesty's Ships Adventure and Beagle between the years 1826 and 1836, describing their examination of the southern shores of South America, and the Beagle's circumnavigation of the globe. Journal and remarks. 1832-1836,* London, Henry Colburn.

DARWIN, C. R. (1839b) *The zoology of the voyage of H.M.S. Beagle. Birds Part 3 No. 4,* London, Smith Elder and Co.

EXCOFFIER, L., LAVAL, G. & SCHNEIDER, S. (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online,* 1**,** 47-50.

GOUDET, J. (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Lausanne University, Lausanne, Switzerland.

GOULD, J. (1837) Three Species of the Genus Orpheus, from the Galapagos, in the Collection of Mr. Darwin. *Proceedings of the Zoological Society of London,* 5**,** 27.

GRANT, P. R., CURRY, R. L. & GRANT, B. R. (2000) A remnant population of the Floreana mockingbird on Champion island, Galapagos. *Biological Conservation,* 92**,** 285-290.

HEY, J. & NIELSEN, R. (2004) Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of Drosophila pseudoobscura and D-persimilis. *Genetics,* 167**,** 747-760.

HOECK, P. E. A., BOLLMER, J. L., PARKER, P. G. & KELLER, L. F. (submitted) Differentiation with drift: a spatio-temporal genetic analysis of Galapagos mockingbird populations (Mimus spp.). *Philosophical Transactions of the Royal Society B*.

HOECK, P. E. A., BUCHER, T. B., WANDELER, P. & KELLER, L. F. (2009) Microsatellite primers for the four Galápagos mockingbird species (*Mimus parvulus*, *M. macdonaldi*, *M. melanotis*, and *M. trifasciatus*). *Molecular Ecology Resources,* in press.

LANGELLA, O. (2000) Populations 1.2.30. Laboratoire Populations, Génétique et Evolution, Centre National de la Recherche Scientifique, CNRS UPR9034, Gif Sur Yvette.

NEI, M. (1972) Genetic Distance between Populations. *American Naturalist,* 106**,** 283-&.

O'RYAN, C., HARLEY, E. H., BRUFORD, M. W., BEAUMONT, M., WAYNE, R. K. & CHERRY, M. I. (1998) Microsatellite analysis of genetic diversity in fragmented South African buffalo populations. *Animal Conservation,* 1**,** 85-94.

PEAKALL, R. & SMOUSE, P. E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,* 6**,** 288-295.

SWARTH, H. (1931) The avifauna of the Galapagos Islands. *Occasional Papers of the California Academy of Science,* 18**,** 5-299.

WANDELER, P., HOECK, P. E. A. & KELLER, L. F. (2007) Back to the future: museum specimens in population genetics. *Trends in Ecology & Evolution,* 22**,** 634-642.

WEIR, B. S. & COCKERHAM, C. C. (1984) Estimating F-statistics for the analysis of population structure *Evolution,* 38**,** 1358-1370.

NO SIGN THAT GENETIC DIVERSITY AFFECTS INNATE IMMUNITY OR ECTOPARASITE LOAD IN GALÁPAGOS MOCKINGBIRD POPULATIONS

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ABSTRACT

Low genetic diversity or inbreeding may impair an individual's immune system, render it more susceptible to disease and hence contribute to the extinction risk of small and isolated populations, as often found on islands. So far, surprisingly few studies have assessed the effects of inbreeding on immunocompetence in wild populations. Four allopatric species of mockingbirds in the Galápagos Islands provide a rare opportunity to test this relationship because they show sufficient variation in inbreeding levels among clearly isolated populations. Using 26 microsatellite loci and genetic data from historic and contemporary samples, we calculated short-term and long-term inbreeding in 13 different mockingbird populations and compared them with three different measures of innate immunity and ectoparasite load. We found no significant effect of either measure of population-level inbreeding on natural antibody or complement enzyme titres, heterophil-lymphocyte ratio or mean number of feather lice. Our analyses therefore do not support a link between inbreeding and innate immune response or ectoparasite load in these species. However, statistical power was low and potential confounding by environmental effects could not be ruled out. Overall, natural antibody titres were high suggesting that the mockingbirds have a strong first line of defense, as previously suggested for other island species.

KEYWORDS

Genetic diversity, immunocompetence, birds, Nesomimus, lice, natural antibodies

INTRODUCTION

In recent times, human-induced habitat fragmentation has increased rapidly worldwide and, as a consequence, animal and plant populations are becoming more and more subdivided, resulting in populations of reduced size. Apart from being more susceptible to demographic and environmental stochastic events (Reed, 2004; Shaffer, 1981), small and isolated populations suffer faster loss of genetic diversity through genetic drift and experience an increase in inbreeding due to mating between relatives. Inbreeding can ultimately lead to elevated extinction risks through direct effects of deleterious mutations or by reducing a population's capacity to evolve and respond to environmental changes (Frankham, 1995; Frankham, 1998; Spielman *et al.*, 2004b). Additionally, reduced genetic diversity may increase extinction risks of small populations by making them more vulnerable to infectious diseases (Cleaveland *et al.*, 2002; McCallum, Dobson, 1995; Smith *et al.*, 2009). Inbred individuals and populations have been shown to exhibit a decrease in resistance to parasites (Acevedo-Whitehouse *et al.*, 2003; Cassinello *et al.*, 2001; Coltman *et al.*, 1999; Hedrick *et al.*, 2001; Pearman, Garner, 2005; Puurtinen *et al.*, 2004), to pathogens (e.g. Ross-Gillespie *et al.*, 2007) and lowered immunocompetence (e.g. Reid *et al.*, 2003; Reid *et al.*, 2007; Whiteman *et al.*, 2006), but results from different studies are contradictory (Ivey *et al.*, 2004; Spielman *et al.*, 2004a). Some studies showed that the effect of parasites depended on the genetic background genotype of the host and the parasite species (Haag *et al.*, 2003; Wiehn *et al.*, 2002), whereas others found no correlation between genetic diversity and resistance to pathogens (Gerloff *et al.*, 2003; Giese, Hedrick, 2003; Stevens *et al.*, 1997). To date, direct evidence from wild populations is still scarce despite its particular interest to conservation biology (Ingvarsson, 2001; Keller, Waller, 2002). Studies on the impacts of inbreeding and disease performed in the laboratory under controlled and stable conditions are likely to underestimate the influence of stochastic environmental and demographic factors (Norris, Evans, 2000). Therefore, for both evolutionary and conservation biology (Acevedo-Whitehouse *et al.*, 2003), it is important to obtain more empirical data to evaluate the overall impacts of inbreeding on the ability of populations to cope with

disease under natural environmental conditions (Keller, Waller, 2002; Smith *et al.*, 2009; Spielman *et al.*, 2004a).

Small host populations on isolated islands are particularly susceptible to foreign pathogens, as they have only been exposed to few pathogens during their recent evolutionary history (McCallum, Dobson, 1995). With the increase of global trade and tourism, formerly isolated island systems such as the Galápagos or Hawaii have become connected, resulting in the introduction of pathogens from the mainland (Vargas, Snell, 1997; Wikelski *et al.*, 2004). Disease has been implicated as a major factor leading to population declines and extinctions of avian species on islands, as for example in Hawaii (Van Riper *et al.*, 1986; Van Riper *et al.*, 2002). Although Galápagos is still one of the best preserved archipelagos worldwide, the introduction of alien avian diseases has become a major concern leading to the initiation of a number of avian health survey projects (Gottdenker *et al.*, 2005; Padilla *et al.*, 2006; Parker *et al.*, 2006; Soos *et al.*, 2008; Wikelski *et al.*, 2004). Some introduced diseases such as avian poxvirus or a recently introduced botfly species have already negatively affected the native fauna in Galápagos (Fessl, Tebbich, 2002; Vargas, 1987) and new diseases are expected to arrive (Wikelski *et al.*, 2004) which could have devastating consequences (Gottdenker *et al.*, 2005), especially for critically endangered endemics such as the Mangrove finch (*Cactospiza heliobates*) or the Floreana mockingbird (*Mimus trifasciatus*).

The Galápagos mockingbirds

In this study, we determined levels of genetic diversity or inbreeding and their effects on several immune measures in different mockingbird species and populations in the Galápagos. Four endemic, allopatrically living mockingbird species are recognized in the Galápagos (Harris, 1974). Three species occur solely on one major island each and/or their satellite islands, namely *Mimus macdonaldi*, *M. trifasciatus* and *M. melanotis*, whereas *M. parvulus* is distributed throughout most other islands of the archipelago (Fig. 1). *M. trifasciatus* today is restricted to only two small satellite islands which have become genetically isolated (Hoeck *et al.*, in press-a) since the disappearance of the

connecting, large mockingbird population on Floreana at around 1880 following habitat alterations with human colonization and the introduction of mammalian predators (Curry, 1986). Pronounced differences in average inbreeding levels have been found among mockingbird species and populations: genetic diversity correlates strongly with island size and between-island population structure is high (Hoeck *et al.*, in press-b). Hence, these isolated populations of different size provide an ideal natural system to study the effects of genetic diversity on different immune measures. Furthermore, genetic data from historical samples allowed the detection of changes in genetic diversity over the last 100 years (Hoeck *et al.*, in press-b) and, hence, allow differentiating between recent inbreeding vs. historically low levels of genetic diversity. This is of interest because populations that have undergone historical processes of inbreeding may have successfully purged much of their genetic load (Crnokrak, Barrett, 2002), resulting in a weaker heterozygosity-fitness relationship (Reed, Frankham, 2003).

Innate immunity

The avian immune system relies on three major defense mechanisms to resist infection by parasites and disease, namely innate immunity, and humoral and cell-mediated acquired immunity (Cheng, Lamont, 1988). All three components have been shown to be under genetic control (Sarker *et al.*, 2000) and could hence be affected by inbreeding. An individual's immunocompetence is based on these three defense components and is defined as its ability to prevent or control infection by pathogens and parasites (Norris, Evans, 2000). Correlations between various indices of immune function and resistance to specific diseases appear to be generally pathogen-dependent and higher levels of one component of the immune system need not imply greater overall resistance (Adamo, 2004; Matson, 2006). Therefore, strong arguments for simultaneous measurement of multiple immune parameters have been put forward (Adamo, 2004; Keil *et al.*, 2001).

Here, we tested the innate immunity in populations of all four Galápagos mockingbird species by counting different types of white blood cells (leucocytes), and the innate humoral immune response by assessing natural antibody and complement enzyme activity. Innate defenses are constitutive and induced rapidly and therefore most

important against first exposures to pathogens and quickly growing infections, resulting in non-specific defense reactions such as inflammatory reaction or phagocytosis. The typical response to infectious or inflammatory diseases in birds is an increase in the total white blood cell (leukocyte) count, mainly caused by an increase in the number of heterophils and/or lymphocytes. An index of the relative abundances of lymphocytes and heterophils is the heterophil-lymphocyte (H:L) ratio, which offers general information about infection status and is widely used to estimate stress in poultry (e.g. Gross, Siegel, 1983; Maxwell, 1993) and wild birds (Tompkins *et al.*, 2006). Heterophils are phagocytic cells and high numbers can indicate either inflammation or stress (e.g. bacterial infections result in a heterophil increase; Fudge, 2000).

Although innate immunity mediated by phagocytic and bactericidal white blood cells represents a potent first line of defense against pathogens, natural antibodies (NAbs) provide a supplementary or alternative defense that can also be deployed initially in response to invading organisms (Zouali, 2001). Their various affinities for multiple antigens include pathogens and toxins (Carroll, Prodeus, 1998; Ochsenbein *et al.*, 1999) and the ability to kill pathogens *in vitro* (Ochsenbein *et al.*, 1999) and *in vivo* (Belperron, Bockenstedt, 2001). Taken together, findings so far suggest a role for NAbs in limiting the initial pathogen burden prior to the development of adaptive immune response (Ochsenbein *et al.*, 1999). NAbs are constitutive and, unlike adaptive defenses, do not first have to develop a memory of specific antigens (e.g. Ochsenbein, Zinkernagel, 2000). As they are directly encoded in nuclear DNA (Belperron, Bockenstedt, 2001) and NAb levels have been shown to respond to selection on other immune components in chickens (Parmentier *et al.*, 2004), genetic differences may influence their titres. NAb levels may hence covary with genetic diversity, as has been shown for other immunologically important proteins in vertebrates such as MHC (Miller, Lambert, 2004). Furthermore, NAb response is hypothesized to predict the strength of the adaptive immune response (Kohler *et al.*, 2003), providing a link between the innate and acquired humoral immunity (Lammers *et al.*, 2004; Ochsenbein, Zinkernagel, 2000). Together with the complement enzymes, NAbs initiate the complement enzyme cascade which eventually leads to cell lysis (Carroll, Prodeus, 1998). Agglutination arises from NAbs only whereas lysis reflects an interaction between NAbs and complement enzymes. In a hemolysis-hemagglutination

test agglutination and lysis titres can be determined, providing an index of the strength of the constitutive innate immune system with higher scores indicating more effective immune responses (Matson *et al.*, 2005). As NAbs and complement have been shown to only weakly positively correlate (Matson 2005), both measures provide valuable, independent information about innate immunocompetence.

Ectoparasite load

Parasites are known to exert strong selection pressures on their hosts through exploitation of host resources (Marshall, 1981), but also indirectly through pathology and activation of the immune system (Wikel, Alarcon-Chaidez, 2001). Ectoparasites encounter host immune defenses when they feed on blood or living tissue (Marshall, 1981) and therewith stimulate a spectrum of different immune responses that potentially impair their development or even kill them (Wikel, Alarcon-Chaidez, 2001). A link between genetic diversity, immunity and ectoparasite abundance has been found e.g. in the Galápagos hawk (Whiteman *et al.*, 2006), whereas no correlation between genetic diversity and parasite load was detected in other studies, e.g. in New Zealand Saddlebacks (Taylor, Jamieson, 2007). Here, we determined ectoparasite load of two louse lineages in order to estimate parasite infestation of mockingbirds and test for a potential relationship between genetic diversity and parasite burden.

We investigated the relationship between genetic diversity and immunocompetence on a population level using two different measures of Fst as estimates of population-level inbreeding. We made the following predictions: a) more inbred populations should show lower average innate and humoral immune response and have a higher ectoparasite load relative to outbred populations, and b) populations that experienced relatively recent, fast inbreeding are expected to show lower immunocompetence and defense against ectoparasites than populations with a long history of low genetic diversity.

This study is unique in combining genetic data from historic and modern samples with several estimates of immunocompetence in natural populations of varying size. Our

results not only extend our knowledge on the interactions between genetic diversity and disease susceptibility, but also establish species-specific baseline immunocompetence and ectoparasite infestation estimates for the mockingbirds, which may be interesting with respect to the conservation management of the endangered Floreana mockingbird.

MATERIALS AND METHODS

Sample collection

We visited the Galápagos Islands from 2006-2008 and collected samples on 11 different islands and at 13 different locations (Fig. 1 & Table 1). On St. Cruz and San Cristóbal, we obtained samples from two different locations, separated by approx. 10 km and 35 km, respectively. We distinguished between the two locations on the same island as previous genetic analyses detected substantial within-island genetic differentiation (Hoeck *et al.*, in press-b). Four islands (Champion, Gardner-by-Floreana, Española and St. Cruz) were visited in all three years to account for variation between years in the parameters estimated here.

Mockingbirds were captured in potter traps and, in some areas, also in mist nets and bled immediately after capture. Blood samples were obtained by a small puncture of the wing vein collecting between 20-140 µl of blood (less than 1% of the body weight of a bird) in heparinized microtubes. Blood sampling was usually finished within 2-4 minutes after capture (mean: 3.5 min). The blood was transferred immediately into Eppendorf tubes for centrifugation on site. Small drops of blood were used to produce two blood smears and one drop was transferred onto filter paper treated with 0.5 M EDTA for later DNA extraction. Filter blood samples were kept dry until DNA extraction. Blood smears were air-dried and fixed and stained with Diff-Quick solution (Medion Diagnostics GmbH, Düdingen, Switzerland). Whole blood was centrifuged in the field and plasma was transferred into a liquid nitrogen container where it was kept frozen until used for the hemolysis-hemagglutination assay in the lab in Switzerland.

Ectoparasite collection and counting

To quantify ectoparasite load, we dust-ruffled a subset of mockingbirds from all populations (Table 1) following the method described in Walther and Clayton (1997). We used pyrethron powder (non-toxic to birds; 0.3% natural flower-extract pyrethrum and 1% piperonyl butoxid; Vetyl-Chemie GmbH, Germany) and applied 0.7g of insecticide to the plumage of the birds (all feather tracts except the head). Dust-ruffling time was kept short to reduce stress for the birds under the hot and sunny field conditions. Dusting was performed for 2.5 min, followed by 1 min of incubation and 2.5 min ruffling over a clean plastic tray to extract ectoparasites. Ectoparasites were stored in 97% ethanol until they were counted and identified in the lab. Although dust-ruffling not only yielded lice, but also mites and other small organisms occurring in the plumage, only adult and nymphal lice were considered for further analyses. They were the largest and clearly most abundant parasites detected (77% of all organisms detected in the plumage) and are hypothesized to potentially cause immune reactions due to their blood-sucking behavior (Wikel, Alarcon-Chaidez, 2001). Identification of louse genera was done by Vincent Smith, Natural History Museum, UK. Lice belonged to two different genera, *Brueelia galapagensis* (Ischnocera) and *Myrsidea sp.* (Amblycera). Because *Brueelia* belongs to the feather-chewing lice, it is unlikely to have a direct effect on the birds' immune system (Moller, Rozsa, 2005), whereas *Myrsidea* lice may cause an immune reaction through their tissue- and blood-feeding behavior (Moller, Rozsa, 2005) typical for amblyceran lice (Marshall, 1981). However, because feather-chewing lice are also known to have negative effects on host fitness by damaging feathers, which compromises e.g. thermoregulatory ability (Booth *et al.*, 1993) or reduces survivorship (Clayton *et al.*, 1999), and might select on behavioral or physical host defenses that may be sensitive to host genetic diversity (Whiteman *et al.*, 2006), we included both *Myrsidea* and *Brueelia* lice in our analyses.

White blood cell counts

We estimated innate immunocompetence by counting different types of white blood cells (WBCs) in blood smears on microscope slides which were examined under 1000 x magnification with oil immersion. The proportion of different types of white

blood cells (lymphocytes, heterophils, eosinophils, basophils and monocytes) was assessed on the basis of an examination of a total of 100 WBCs. All counts were performed by the same person (PEAH). A cross-sectional method of slide scanning was used to prevent scanning the same area twice (Reauz *et al.*, 1999). To estimate variation within the same smear, we repeated the count of 100 WBCs for 20 different individuals. Additionally, to estimate variation between two smears taken from the same individual at the same time, we also counted 100 WBCs in the second smear of 22 different individuals. We only used data for lymphocytes and heterophils as the most numerous immune cells in our analyses.

Hemolysis-hemagglutination assay

Agglutination and lysis titers were assessed with a hemolysis-hemagglutination assay as described in Matson *et al.* (2005), which measures the interaction between NAbs and antigens in rabbit red blood cells (agglutination) and the action of complement from the amount of haemoglobin released from lysis of rabbit erythrocytes (lysis). Plasma was serially diluted twofold with saline in a 96-well assay plate and incubated with rabbit red blood cells (Harlan Laboratories UK Ltd.) for 90 min at 37°C. Because we only obtained small volumes of plasma samples from most mockingbirds, we omitted the first, undiluted row of plasma in the assay, hence working with plasma volumes of 25 µl and a series of dilutions ranging from 1/2 to 1/4096. Samples were placed on plates haphazardly and, as a control, a chicken plasma sample (Harlan Laboratories UK Ltd.) was added onto each batch of six plates that were processed at the same time on the same tray. After completion of the test, we determined the dilution step at which either the agglutination or lysis reaction stopped (titer score) and took digital images. As a control, titer scores were confirmed a few days later using the digital images only. All scoring was carried out blindly with respect to the identity of the individual birds and always performed by the same person (PEAH). Repeat tests were done on 16 individuals on two different plates to estimate within-individual repeatability among different assay plates. To account for differences between plates processed at different times, all mockingbird lysis and agglutination scores were corrected for the chicken control that was run at the

same time by substracting the score of the chicken sample from the mockingbird sample score.

DNA extraction and microsatellite analysis

Extraction of DNA from blood on filter paper was performed as described in Hoeck *et al.* (2009). DNA was amplified at 26 microsatellite loci, 6 of which (MpAAT18, 25, 26, 45, 83 and 96) were developed in *Mimus polyglottos* (Hughes, Deloach, 1997) with primers for MpAAT45 and MpAAT83 redesigned in our lab (Hoeck *et al.*, 2009). The other 20 microsatellite loci (Nes01, 03, 04, 05, 06, 07, 08, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22 and 23) were designed in *Mimus parvulus* in our lab (Hoeck *et al.*, 2009). PCR conditions were used as published previously and loci were amplified in six independent multiplex reactions (Panel A-D, Hoeck *et al.*, 2009); Panel E with MpAAT18, 25, 45 and 83; Panel F with MpAAT26 and 96 under the same PCR conditions as Panel B & C). Fragment analyses were done on a 3730 DNA Analyser using Gene-Scan-500 LIZ size standard (ABI) and Genemapper v.4 software (ABI) followed by manual proofreading of genotypes.

Although male and female mockingbirds vary slightly in body size, the sexes are indistinguishable based on plumage, hence morphology-based sexing can prove tricky. Therefore, we performed molecular sexing by amplifying the CHD-W and CHD-Z genes (Griffiths *et al.*, 1998) using redesigned primers as described in Hoeck *et al.* (2009) to be able to control for sex in our analyses.

Genetic diversity and inbreeding estimates

Hardy-Weinberg equilibrium for each locus was tested with allele randomizations within samples (1'000 permutations per test) and overall samples (10'000 permutations) using FSTAT 2.9.3.1 package (Goudet, 2001) and adjusting for multiple comparisons using Bonferroni corrections. Genotypic equilibrium between all pairs of loci in each population was tested using G-statistics with Bonferroni corrections and 84'500 permutations in FSTAT. Within population genetic diversity was estimated using standard genetic parameters such as number of alleles (NA), allelic richness (AR, standardized to the smallest sample size) and expected heterozygosity (He) as calculated

in FSTAT. As NA and AR correlated strongly with He (NA: r^2=0.89, p<0.001; AR: r^2=0.94, p<0.001), we decided to use He as a measure for population genetic diversity in our analyses.

To distinguish between recent (i.e. short-term) and long-term inbreeding in the different populations, we calculated two different measures of Fst. As a measure of recent inbreeding we used the changes in genetic diversity over the last century experienced by each population, i.e. the change in allele frequencies within each island since 1906 ("temporal Fst", Table 1; Hoeck *et al.*, in press-b). To quantify long-term inbreeding, i.e. levels of inbreeding that arose since the last common ancestral population, we calculated population-specific Fst values (Biebach, Keller, in press) using the software 2mod (Ciofi *et al.*, 1999). The programme calculates the relative likelihood of two demographic models using coalescent theory and Markov Chain Monte Carlo (MCMC) simulations: a gene flow model that assumes that the gene frequencies within islands are determined by a balance between genetic drift and immigration, and a drift model that assumes independent populations diverging from an ancestral panmictic population by drift only (Ciofi *et al.*, 1999). We selected the "infer model" mode of the programme and ran it with 500'000 iterations to get estimates of population-specific Fst.

Heterozygosity-heterozygosity correlations

The use of microsatellite markers to infer inbreeding has been questioned because microsatellite heterozygosity is unlikely to be a good predictor of the inbreeding coefficient of individuals in all cases (Balloux *et al.*, 2004; DeWoody, DeWoody, 2005; Hansson, Westerberg, 2002; Slate *et al.*, 2004). We therefore estimated the degree to which heterozygosity is correlated across unlinked markers following the method described in Balloux *et al.* (2004). The method is based on the assumption that if microsatellite and genome-wide heterozygosity are correlated, then heterozygosity estimated from one set of microsatellites should be positively correlated with heterozygosity from an independent set of microsatellites from the same individual ('heterozygosity–heterozygosity correlations'). Following Balloux *et al.* (2004) we randomly split the 26 loci into two sets of 13 independent loci and performed the analysis, a) for each population separately to estimate heterozygosity-heterozygosity

correlations on the individual level within a population, and b) in a separate analysis, including all populations and individuals to estimate correlations on the population level, both times using 1'000 iterations to calculate the distribution of the correlation coefficients between the two sets of loci. We detected high correlations when including all individuals and populations in the analysis (mean: 0.65 ± s.d. 0.03), but low or no correlation when doing analyses for each population separately (mean r^2 values ranging from -0.24 to 0.16 with distributions including 0). We therefore did not perform any analyses on the individual level, i.e. we did not include individual multilocus heterozygosity in any of our statistical models, but restricted our inbreeding – immunocompetence analyses to the population level.

Statistical analyses

Repeatabilities to test for within and among individual variances in H:L ratios, as well as lysis and agglutination scores were performed according to Lessells & Boag (1987). To stabilize variances and reach more normal distributions, Box-Cox transformations were performed for the response variables H:L ratio (lamda=0) and number of feather lice (lambda=0.25; Box, Cox, 1964).

Our aim was to assess the relationship between inbreeding and immunocompetence estimates. However, first we carried out an analysis to correct for effects that may also influence our immune and parasite estimates and hence lead to differences between islands. To this end, we performed individual-based generalized linear mixed model (GLMM) analyses, i.e. including all sampled individuals, to account for any sex, year or species effects. We included sex, year and species as fixed effect variables, species nested within island as random effect, and performed separate analyses for each of the four response variables, i.e. agglutination, lysis, H:L ratio and number of feather lice, to obtain the least squares means for these variables. We then used these least squares means (as measures containing a correction for sex, year and species effects) to investigate the relationship between levels of inbreeding (temporal and population-specific Fst) and immunocompetence. We carried out population-level generalized linear models (GLM) analyses using temporal and population-specific Fst as

fixed effect variables and the least squares means from the individual-based analyses as response variables, again performing a separate analysis for each of the four responses. Qualitatively, using the least squares means instead of the true population means for the four response variables did not provide any differing results (data not shown). Therefore, in our final models, we used the true population means for agglutination, lysis, H:L ratio and number of feather lice. We also performed analyses including mean population He as a further explanatory variable, but omitted it in our final analyses because He and population-specific Fst were highly correlated (r^2=0.96, p<0.0001) and He did not add any additional information to our model.

The four different response variables did not correlate (all p-values > 0.12), except for lysis and number of feather lice which correlated positively at the population level (r^2=0.36, p=0.023), but not at the individual level (r^2=0.001, p=0.78). We therefore did not expect to have any problems of co-linearity in our GLM analyses. Not all information was available for all individuals, and sample sizes therefore varied slightly among analyses (Table 1 & 2). All analyses were performed using the software JMP Version 8 and SAS Version 9.1 (SAS Institute Inc., Cary, NC) with significance set to alpha = 0.05.

RESULTS

Ectoparasites

We dust-ruffled 196 individuals from all 13 populations (Table 1) and collected a total of 1482 *Myrsidea* and 133 *Brueelia* individuals. *Myrsidea* occurred on all islands whereas *Brueelia* lice were only found in samples from Española, Gardner-by-Española, Gardner-by-Floreana, Marchena, Rábida and St. Fé (Fig. 2a). The mean number of *Myrsidea* lice in the plumage of a mockingbird was 7.6 (± s.d. 6.5), ranging from 0 to 35 lice, and *Myrsidea* showed a prevalence of 95.4%. On the six islands where *Brueelia* occurred, mean infestation of *Brueelia* was 1.26 (± s.d. 1.7), ranging from 0 to 10 lice, and prevalence was 41%. Because parasite abundance has been shown to correlate positively with island size in Darwin's finches (Lindstrom *et al.*, 2004), we also tested for

an effect of island size on mean feather louse load in the different populations but found no significant correlation (r^2=0.21, p=0.12).

White blood cell counts

Within-smear repeatability was 82.5% ($F_{19,20}$=12.9, p<0.0001) and between-smear repeatability 84.7% ($F_{21,22}$ =16.4, p<0.0001), demonstrating that our white blood cell counts were reliable. Mean H:L ratio varied considerably among islands with the two *M. macdonaldi* populations showing the highest values (Table 1 & Fig. 2b).

Hemolysis-hemagglutination assay

Repeat tests on the 16 individuals that were analyzed twice (but on different assay plates) showed that repeatability was 8% for the hemagglutination reaction ($F_{15,16}$=1.24, p=0.33), whereas it was 58% for the hemolysis reaction ($F_{15,15}$=4.57, p=0.003). Hence, most of the measuring variance for agglutination lies within individuals, i.e. repeatability for agglutination was very low.

Agglutination scores of individuals ranged from 3.5 to 11 (the maximum possible) with an overall mean score of 8.8 (± s.d. 1.2) and lysis ranged from 0 to 7 with a mean of 3.4 (± s.d. 1.2). Overall, lysis and agglutination scores varied among populations (Fig. 2c & 2d), with a difference of nearly two scores in mean agglutination titres and 1.5 scores in mean lysis titres (Table 1). It is interesting to note that no lysis occurred in any of the samples collected on Rábida (Table 1).

Genotyping and genetic diversity estimates

We genotyped blood samples from a total of 400 individuals (Table 1). Successful genotypes were obtained for all individuals and loci except for one individual which could not be genotyped at one single locus. All 26 loci were in Hardy-Weinberg equilibrium (HWE) in all populations except in four cases: MpAAT96 and Nes03 in Gardner-by-Floreana, Nes04 in Santiago and Nes22 in Marchena which showed an excess of homozygotes. Genotypic disequilibrium was only detected for two locus pairs in one single population (MpAAT96 x Nes03 and Nes05 x Nes01 in Gardner-by-Floreana).

The mean number of alleles per locus was 9.5 and ranged from 3 to 18 alleles. The mean number of alleles per population was 91 (range: 32-154, Table 1), mean allelic richness was 3 (range: 1.18-4.69, Table 1) and mean He 0.4 (range: 0.06-0.65, Table 1).

Population-specific Fst values were very high, ranging from 0.1 for the least inbred population on St. Cruz to 0.9 for the most inbred population on Champion (Table 1). As expected Fst decreased significantly with island size (r^2=0.68, p<0.001; Fig. 3). Population-specific Fst correlated significantly (r^2=0.52, p=0.006) with temporal Fst (Hoeck *et al.*, in press-b). However, as the two Fst values represent two different measures of inbreeding, i.e. long-term inbreeding versus short-term, they were both kept in the main analyses.

Inbreeding, immunocompetence and ectoparasites

We found no significant effect of population-specific or temporal Fst on agglutination or lysis score, H:L ratio or number of feather lice (Table 2 & Fig. 2a-d). Hence, contrary to our prediction, we detected no evidence for either of the two inbreeding estimates to affect any of our immunocompetence estimates or ectoparasite load. These results did not change when we only included the blood-sucking *Myrsidea* lice in our model, and hence omitted *Brueelia* (p-values >0.47), or added He as an additional fixed effect (all p-values for He >0.22, data not shown). However, statistical power for the analyses was low, as reflected by the large confidence intervals of the parameter estimates (Table 2).

DISCUSSION

Inbreeding, immunocompetence and ectoparasites

As previous studies support a link between reduced genetic variation and susceptibility to pathogens (e.g. Acevedo-Whitehouse *et al.*, 2003; Acevedo-Whitehouse *et al.*, 2005; Cassinello *et al.*, 2001; Coltman *et al.*, 1999; Coulson *et al.*, 1998; Haag *et al.*, 2003; Hawley *et al.*, 2005; Luong *et al.*, 2007; MacDougall-Shackleton *et al.*, 2005) or immunocompetence (e.g. Hawley *et al.*, 2005; Reid *et al.*, 2003; Whiteman *et al.*,

2006), we predicted a positive relationship between levels of inbreeding and feather louse load as well as H:L ratio, and a negative relationship with natural antibody (agglutination) and complement enzyme (lysis) titres. However, we detected no significant relationships between different immune estimates and either of our two inbreeding estimates (Fig. 2 & Table 2). There are several possible explanations for a lack of such correlations:

a) A weakness of an observational study design, as used here, is the small sample size (on a population level) and consequently low statistical power, as reflected in the large confidence intervals (Table 2). However, our sample size was larger than in a comparable study that did detect significant effects of genetic diversity on innate immunity and parasite load (Whiteman *et al.*, 2006). It is likely that some of the observed variation in ectoparasite infestation or immune estimates detected here will be due to spatial and temporal environmental variability. Hence, environmental effects might have confounded our analyses, even though we corrected for effects of sampling in different years in our analyses. However, if such (environmental) variability is large in comparison to the heterozygosity-associated signal, then the signal will be difficult to detect statistically. The low repeatability detected for two of our immunocompetence measures (lysis and especially agglutination) also weakens the strength of our results. Such a low repeatability is surprising as the hemolysis-hemagglutination test has previously been shown to be a highly repeatable assay (Matson *et al.*, 2005).

b) A relationship between inbreeding and parasite infestation might be context dependent, i.e. parasite resistance may only influence fitness in some occasions, e.g. when nutritional or environmental conditions are stressful for a host (Arcese, 2003). As organisms need to balance costs and benefits differently, dependent upon life history context and environmental conditions, immune responses that convey costs and depletion of energy that could be used otherwise (Nelson *et al.*, 2002), are likely to be traded-off against other traits (e.g. Hasselquist *et al.*, 1999; Nelson *et al.*, 2002; Zuk, Stoehr, 2002). Hence, we may have been unable to detect potential correlations between inbreeding and immunocompetence if these only become significant under stressful conditions.

Furthermore, differences in levels of inbreeding might only show an effect on immunocompetence in an immunologically challenging environment. The fitness effects of immunosuppression necessarily depend on the intensity of pathogen threats, which may not be sufficient in isolated and relatively pathogen-poor environments of island systems, such as the Galápagos (Frankham, 1997; McCallum, Dobson, 1995; Wikelski *et al.*, 2004). Hence, immunologically relevant genetic diversity may have been lost through mutation and drift if benefits from such diversity are reduced (Frankham, 1997).

c) As the Galápagos Islands are exposed to strong environmental variation ranging from very dry La Niña to extremely wet El Niño conditions, and pathogen pressure is known to fluctuate in a changing environment (Moyer *et al.*, 2002a; Salam *et al.*, 2009), mockingbirds might also have adapted their innate immune systems to this wide range of conditions and purged some of their genetic load (Crnokrak, Barrett, 2002). Deleterious alleles that contribute to inbreeding depression in the immune traits investigated here may have been purged, resulting in the absence of a relationship between inbreeding and immunocompetence, as previously suggested (e.g. Visscher *et al.*, 2001; Wiehn *et al.*, 2002). If purging of recessive deleterious alleles obscured a potential heterozygosity-fitness relationship (Reed, Frankham, 2003), we may have expected that populations with relatively recent, fast inbreeding and strong genetic drift, such as the two small *M. trifasciatus* populations (Hoeck *et al.*, in press-b), would show lower immunocompetence or more ectoparasites than populations with a long history of low genetic diversity (such as the populations on St. Fé or Española). However, we did not detect any such pattern (Table 2 & Fig. 2). Temporal Fst (i.e. an estimate for recent inbreeding) may not correlate with any of the immune or parasite measures because the two *M. trifasciatus* populations may have already spent enough generations in isolation to reduce inbreeding effects (i.e. an estimated approx. 50-100 generations; Hoeck *et al.*, in press-a). Purging within this time frame seems likely as, for example, in a study on *Drosophila* it has been shown that as few as 20 generations after an inbreeding event were sufficient to significantly reduce the amount of inbreeding depression (Fowler, Whitlock, 1999).

d) Genetic variation in immune traits or resistance to parasites could be associated with specific alleles rather than inbreeding or heterozygosity *per se* (Spielman *et al.*, 2004a). For example, it has been found that specific MHC alleles affect the regulation of immune responses or parasite resistance (Bonneaud *et al.*, 2005; Eizaguirre *et al.*, 2009). We suggest that a candidate-gene approach, such as MHC, might be more fruitful for future research to detect possible gene-resistance correlations in mockingbirds.

e) If different measures of immunity are traded-off against each other, there may be a relationship between levels of inbreeding and other arms of the birds' immune system not covered by this study, such as the adaptive, cell-mediated or humoral immune response. If the loss of genetic diversity impairs acquired immunity (e.g. specific antibody response), a shift in the functional balance could result in greater reliance on innate immunity. Due to restrictions given by the conditions in the field and work with protected and endangered species, we were unable to assess acquired immunity or total parasite infestation. Hence, we can also not rule out the possibility for inbreeding to have an effect on endoparasite resistance. Ectoparasites are only a subset of the parasites that infest a host and as they do not occur within the host's body, such as endoparasites, ectoparasites are less likely to respond to subtle differences in the immune response (Wakelin, 1996).

f) Ectoparasite resistance and the innate immune traits measured here may simply not be affected by inbreeding, i.e. there may not be a link between genetic diversity and fitness for these traits in the Galápagos mockingbirds. In a recent review on heterozygosity-fitness correlations in animal populations, it was detected that 24% of the correlations were non-significant (Chapman *et al.*, 2009), indicating that such results are common. Although positive heterozygosity-fitness correlations have been widely reported (for reviews see e.g. Crnokrak, Roff, 1999; Keller, Waller, 2002; Reed, Frankham, 2003), some fitness traits, such as parasite resistance, are in many cases unrelated to genetic diversity (Coltman, Slate, 2003; Cote *et al.*, 2005; Hedrick *et al.*, 2001; Poulin *et al.*, 2000a; Poulin *et al.*, 2000b; Stevens *et al.*, 1997; Taylor, Jamieson, 2007). A relationship between parasite resistance and genetic diversity in natural

populations may be absent when the variation in infective ability within particular parasite species is of little consequence to host resistance. Such a low selection pressure could be expected in the case of feather-chewing lice and blood-sucking lice that occur in low numbers.

g) In this study, we only caught adult birds that walked into potter traps or flew into mist nets. Severe parasite infestation or disease might quickly lead to mortality or immobility and thus, sick birds would probably have gone undetected. Additionally, we would not have detected selection that occurs early in life because we did not sample any nestlings or fledglings. We can therefore not rule out the possibility that inbreeding may affect immunocompetence early in life, on which selection may then act.

Ectoparasites/ feather lice

Amblyceran lice (*Myrsidea*) were much more abundant than ischnocerans (*Brueelia*), a pattern generally observed when these suborders co-occur on the same host (Whiteman, Parker, 2004 and references therein). Interestingly, however, *Brueelia* was not detected in any of the ectoparasite samples collected on the large islands of Isabela, San Cristóbal, Santiago and Santa Cruz, and mean feather louse load did not correlate with island size. This is in contrast to general island biogeography models which predict hosts living on larger islands to be exposed to a higher diversity of parasites (MacArthur, Wilson, 1967), and findings in wild bird populations that support this prediction (e.g. Lindstrom *et al.*, 2004). Also, these larger Galápagos islands have the highest elevation and therewith humid zones, an environment found to be beneficial for ectoparasites (Moyer *et al.*, 2002b). It is unlikely that the lack of *Brueelia* on these larger islands is due to competition among ectoparasite species because we did not detect any other louse species other than *Myrsidea* in the plumage of birds from these islands and total louse load did not increase with island size (data not shown). Confirming and explaining the absence of *Brueelia* on the larger islands remains subject to further investigations.

Immunocompetence measures

Various stressors, such as infectious disease or parasitemia, lead to an increase in leucocyte numbers, especially heterophils and lymphocytes. Therefore, the H:L ratio has been suggested to serve as a measurement of infection status and physiological stress in birds (Gross, Siegel, 1983). Based on the differential WBC count determined here, i.e. the H:L ratio, we cannot infer whether overall leucocyte numbers in the blood were high because we did not determine total WBC concentrations in relation to red blood cell numbers. If inbreeding affects the concentration of both, heterophils and lymphocytes we may not be able to detect a relationship between inbreeding and H:L ratio (Table 2 & Fig 2b). For example, a severely bottlenecked population of New Zealand robins differed in its total lymphocyte and leucocyte numbers from the genetically diverse source population, but showed no difference in its H:L ratio (Hale, Briskie, 2007). We did not find any significant effects of the two measures of inbreeding on mean heterophil or mean lymphocyte numbers in the different populations (Anova, all p-values >0.31). Nevertheless, it may be interesting to determine WBC relative to red blood cell concentrations to distinguish between overall versus relative leucocyte numbers.

As already found in previous studies (Matson *et al.*, 2005; Mendes *et al.*, 2006), we did not detect any correlation between NAb levels and complement-mediated lysis, supporting the general idea that they serve as two independent measures of immunocompetence. However, the low repeatabilities found here caution such a statement. The mean levels of agglutination in the mockingbirds (Table 1) were higher or at the high end of recently reported values from a range of birds (Matson, 2006; Matson *et al.*, 2005; Mendes *et al.*, 2006; Parejo, Silva, 2009), suggesting that Galápagos mockingbirds have high levels of natural antibodies. Levels are comparable to the ones found in another Galápagos endemic, the Galápagos hawk (Whiteman *et al.*, 2006). In contrast to the results obtained from the mockingbirds, however, inbred Galápagos hawk populations harbored lower average and less variable NAb levels than relatively outbred populations (Whiteman *et al.*, 2006), which lead the authors to conclude that the peripheral, inbred hawk populations are vulnerable to disease agents. Furthermore, no lysis reactions were reported for the hawks (Whiteman *et al.*, 2006), as for other species

(Matson, 2006), whereas all of the mockingbird populations showed lysis reactions except for the individuals from Rábida (Table 2). As NAbs play an important role in the initial recognition of foreign particles and support subsequent defense by the complement cascade and the acquired humoral response (Ochsenbein, Zinkernagel, 2000), the high NAb and relatively high complement enzyme titres found here could indicate that the mockingbirds are equipped with a strong first line of defense. Increased innate defenses of insular birds in comparison to their continental relatives have previously been reported and interpreted as a shift in the immune defense strategy towards innate as opposed to acquired immune responses (Matson, 2006). If NAb response does also predict the strength of the adaptive humoral immune response (Kohler *et al.*, 2003; Lammers *et al.*, 2004), this would furthermore suggest that the mockingbirds could have a potent adaptive immune system. Based on the results from this study, however, we are unable to predict how the mockingbirds would cope with new, introduced pathogens known to have caused devastating population declines and extinctions on other islands (e.g. Van Riper, Scott, 2001; Van Riper *et al.*, 1986). This stresses the importance of future avian health surveys and disease control in the Galápagos.

Conclusions

We investigated whether inbreeding affects immunocompetence using many different Galápagos mockingbird populations, varying in size and levels of inbreeding, and covering the range of all four endemic species occurring in the Galápagos. Additionally, we included historic as well as modern genetic data to distinguish between relatively recent versus long-term inbreeding, and examined three different measures of the birds' innate immunocompetence. We found no signs that higher levels of inbreeding result in lower levels of innate immunocompetence or higher numbers of feather lice in the Galápagos mockingbirds. Our statistical power, however, was low. The relatively high levels of agglutination and lysis detected here suggest that the mockingbirds have a strong first line of defense against invading pathogens.

The two endangered Floreana mockingbird (*M. trifasciatus*) populations did not show lower levels of natural antibodies, complement enzymes or higher heterophil-

lymphocyte ratios than the other mockingbird populations investigated here. This suggests that *M. trifasciatus* does not suffer from immunosuppression in comparison to the more outbred and more widely distributed mockingbird species. Whether this is because inbreeding does in fact not affect the Floreana mockingbirds' immunocompetence or whether it is simply a reflection of its isolated state on two very small islets with pristine habitats where new pathogens are less likely to arrive than on inhabited islands, remains subject to investigations currently underway (Deem *et al.*, in prep.).

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REFERENCES

Acevedo-Whitehouse K, Gulland F, Greig D, Amos W (2003) Disease susceptibility in California sea lions. *Nature* **422**, 35-35.

Acevedo-Whitehouse K, Vicente J, Gortazar C*, et al.* (2005) Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Molecular Ecology* **14**, 3209-3217.

Adamo SA (2004) How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour* **68**, 1443-1449.

Arcese P (2003) Individual quality, environment, and conservation. In: *Animal Behavior and Wildlife Conservation* (eds. Festa-Bianchet M, Apollonio M), pp. 271-295. Island Press, Washington, D.C.

Balloux F, Amos W, Coulson T (2004) Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology* **13**, 3021-3031.

Belperron AA, Bockenstedt LK (2001) Natural antibody affects survival of the spirochete Borrelia burgdorferi within feeding ticks. *Infection and Immunity* **69**, 6456-6462.

Biebach I, Keller LF (in press) Inbreeding in reintroduced populations: the effects of early reintroduction history and contemporary processes. *Conservation Genetics*.

Bonneaud C, Richard M, Faivre B, Westerdahl H, Sorci G (2005) An Mhc class I allele associated to the expression of T-dependent immune response in the house sparrow. *Immunogenetics* **57**, 782-789.

Booth DT, Clayton DH, Block BA (1993) Experimental Demonstration of the Energetic Cost of Parasitism in Free-Ranging Hosts. *Proceedings of the Royal Society of London Series B-Biological Sciences* **253**, 125-129.

Box GEP, Cox DR (1964) An Analysis Of Transformations. *Journal Of The Royal Statistical Society Series B-Statistical Methodology* **26**, 211-252.

Carroll MC, Prodeus AP (1998) Linkages of innate and adaptive immunity. *Current Opinion in Immunology* **10**, 36-40.

Cassinello J, Gomendio M, Roldan ERS (2001) Relationship between coefficient of inbreeding and parasite burden in endangered gazelles. *Conservation Biology* **15**, 1171-1174.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC (2009) A quantitative review of heterozygosity-fitness correlations in animal populations. *Molecular Ecology* **18**, 2746-2765.

Cheng S, Lamont SJ (1988) Genetic-Analysis of Immunocompetence Measures in a White Leghorn Chicken Line. *Poultry Science* **67**, 989-995.

Ciofi C, Beaumont MA, Swingland IR, Bruford MW (1999) Genetic divergence and units for conservation in the Komodo dragon Varanus komodoensis. *Proceedings of the Royal Society of London Series B-Biological Sciences* **266**, 2269-2274.

Clayton DH, Lee PLM, Tompkins DM, Brodie ED (1999) Reciprocal natural selection on host-parasite phenotypes. *American Naturalist* **154**, 261-270.

Cleaveland S, Hess GR, Dobson AP*, et al.* (2002) The role of pathogens in biological conservation. In: *The Ecology of Wildlife Diseases* (eds. Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP), pp. 139-150. Oxford University Press.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM (1999) Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* **53**, 1259-1267.

Coltman DW, Slate J (2003) Microsatellite measures of inbreeding: A meta-analysis. *Evolution* **57**, 971-983.

Cote SD, Stien A, Irvine RJ*, et al.* (2005) Resistance to abomasal nematodes and individual genetic variability in reindeer. *Molecular Ecology* **14**, 4159-4168.

Coulson TN, Pemberton JM, Albon SD*, et al.* (1998) Microsatellites reveal heterosis in red deer. *Proceedings of the Royal Society of London Series B-Biological Sciences* **265**, 489-495.

Crnokrak P, Barrett SCH (2002) Perspective: Purging the genetic load: A review of the experimental evidence. *Evolution* **56**, 2347-2358.

Crnokrak P, Roff DA (1999) Inbreeding depression in the wild. *Heredity* **83**, 260-270.

Curry RL (1986) Whatever happened to the Floreana mockingbird? *Noticias de Galapagos* **43**, 13-15.

Deem SL, Hoeck PEA, Cruz M, Merkel J, Parker PG (in prep.) Disease risks associated with the re-introduction of the Floreana mockingbird (Mimus trifasciatus) to its island of origin in the Galapagos.

DeWoody YD, DeWoody JA (2005) On the estimation of genome-wide heterozygosity using molecular markers. *Journal of Heredity* **96**, 85-88.

Eizaguirre C, Yeates SE, Lenz TL, Kalbe M, Milinski M (2009) MHC-based mate choice combines good genes and maintenance of MHC polymorphism. *Molecular Ecology* **18**, 3316-3329.

Fessl B, Tebbich S (2002) Philornis downsi - a recently discovered parasite on the Galapagos archipelago - a threat for Darwin's finches? *Ibis* **144**, 445-451.

Fowler K, Whitlock MC (1999) The variance in inbreeding depression and the recovery of fitness in bottlenecked populations. *Proceedings of the Royal Society B-Biological Sciences* **266**, 2061-2066.

Frankham R (1995) Inbreeding and Extinction - a Threshold Effect. *Conservation Biology* **9**, 792-799.

Frankham R (1997) Do island populations have less genetic variation than mainland populations? *Heredity* **78**, 311-327.

Frankham R (1998) Inbreeding and extinction: Island populations. *Conservation Biology* **12**, 665-675.

Fudge AM (2000) *Laboratory Medicine: Avian and Exotic Pets* W. B. Saunders Company, Philadelphia, PA.

Gerloff CU, Ottmer BK, Schmid-Hempel P (2003) Effects of inbreeding on immune response and body size in a social insect, Bombus terrestris. *Functional Ecology* **17**, 582-589.

Giese AR, Hedrick PW (2003) Genetic variation and resistance to a bacterial infection in the endangered Gila topminnow. *Animal Conservation* **6**, 369-377.

Gottdenker NL, Walsh T, Vargas H*, et al.* (2005) Assessing the risks of introduced chickens and their pathogens to native birds in the Galapagos Archipelago. *Biological Conservation* **126**, 429-439.

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3), Lausanne University, Lausanne, Switzerland.

Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. *Molecular Ecology* **7**, 1071-1075.

Gross WB, Siegel HS (1983) Evaluation of the Heterophil Lymphocyte Ratio as a Measure of Stress in Chickens. *Avian Diseases* **27**, 972-979.

Haag CR, Sakwinska O, Ebert D (2003) Test of synergistic interaction between infection and inbreeding in Daphnia magna. *Evolution* **57**, 777-783.

Hale KA, Briskie JV (2007) Decreased immunocompetence in a severely bottlenecked population of an endemic New Zealand bird. *Animal Conservation* **10**, 2-10.

Hansson B, Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology* **11**, 2467-2474.

Harris MP (1974) *The birds of Galapagos* Houghton-Mifflin, New York.

Hasselquist D, Marsh JA, Sherman PW, Wingfield JC (1999) Is avian humoral immunocompetence suppressed by testosterone? *Behavioral Ecology and Sociobiology* **45**, 167-175.

Hawley DM, Sydenstricker KV, Kollias GV, Dhondt AA (2005) Genetic diversity predicts pathogen resistance and cell-mediated immunocompetence in house finches. *Biology Letters* **1**, 326-329.

Hedrick PW, Kim TJ, Parker KM (2001) Parasite resistance and genetic variation in the endangered Gila topminnow. *Animal Conservation* **4**, 103-109.

Hoeck PEA, Beaumont MA, James KE*, et al.* (in press-a) Saving Darwin's muse: evolutionary genetics for the recovery of the Floreana mockingbird. *Biology Letters*.

Hoeck PEA, Bollmer JL, Parker PG, Keller LF (in press-b) Differentiation with drift: a spatio-temporal genetic analysis of Galapagos mockingbird populations (Mimus spp.). *Philosophical Transactions of the Royal Society B*.

Hoeck PEA, Bucher TB, Wandeler P, Keller LF (2009) Microsatellite primers for the four Galapagos mockingbird species (Mimus parvulus, Mimus macdonaldi, Mimus melanotis and Mimus trifasciatus). *Molecular Ecology Resources* **9**, 1538-1541.

Hughes CR, Deloach DM (1997) Developing microsatellites when they are rare: Trinucleotide repeat loci in the northern mockingbird Mimus polyglottos. *Molecular Ecology* **6**, 1099-1102.

Ingvarsson PK (2001) Restoration of genetic variation lost - The genetic rescue hypothesis. *Trends in Ecology & Evolution* **16**, 62-63.

Ivey CT, Carr DE, Eubanks MD (2004) Effects of inbreeding in Mimulus guttatus on tolerance to herbivory in natural environments. *Ecology* **85**, 567-574.

Keil D, Luebke RW, Pruett SB (2001) Quantifying the relationship between multiple immunological parameters and host resistance: Probing the limits of reductionism. *Journal of Immunology* **167**, 4543-4552.

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. *Trends in Ecology & Evolution* **17**, 230-241.

Kohler H, Bayry J, Nicoletti A, Kaveri SV (2003) Natural autoantibodies as tools to predict the outcome of immune response? *Scandinavian Journal of Immunology* **58**, 285-289.

Lammers A, Klomp MEV, Nieuwland MGB, Savelkoul HFJ, Parmentier HK (2004) Adoptive transfer of natural antibodies to non-immunized chickens affects subsequent antigen-specific humoral and cellular immune responses. *Developmental and Comparative Immunology* **28**, 51-60.

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities - a common mistake. *Auk* **104**, 116-121.

Lindstrom KM, Foufopoulos J, Parn H, Wikelski M (2004) Immunological investments reflect parasite abundance in island populations of Darwin's finches. *Proceedings of the Royal Society of London Series B-Biological Sciences* **271**, 1513-1519.

Luong LT, Heath BD, Polak M (2007) Host inbreeding increases susceptibility to ectoparasitism. *Journal of Evolutionary Biology* **20**, 79-86.

MacArthur R, Wilson E (1967) *The Theory of Island Biogeography* Princeton University Press, Princeton.

MacDougall-Shackleton EA, Derryberry EP, Foufopoulos J, Dobson AP, Hahn TP (2005) Parasite-mediated heterozygote advantage in an outbred songbird population. *Biology Letters* **1**, 105-107.

Marshall AG (1981) *The Ecology of Ectoparasitic Insects* Academic Press, London.

Matson KD (2006) Are there differences in immune function between continental and insular birds? *Proceedings of the Royal Society B-Biological Sciences* **273**, 2267-2274.

Matson KD, Ricklefs RE, Klasing KC (2005) A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Developmental and Comparative Immunology* **29**, 275-286.

Maxwell MH (1993) Avian Blood Leukocyte Responses to Stress. *Worlds Poultry Science Journal* **49**, 34-43.

McCallum H, Dobson A (1995) Detecting Disease and Parasite Threats to Endangered Species and Ecosystems. *Trends in Ecology & Evolution* **10**, 190-194.

Mendes L, Piersma T, Hasselquist D, Matson KD, Ricklefs RE (2006) Variation in the innate and acquired arms of the immune system among five shorebird species. *Journal of Experimental Biology* **209**, 284-291.

Miller HC, Lambert DM (2004) Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). *Molecular Ecology* **13**, 3709-3721.

Moller AP, Rozsa L (2005) Parasite biodiversity and host defenses: chewing lice and immune response of their avian hosts. *Oecologia* **142**, 169-176.

Moyer BR, Drown DM, Clayton DH (2002a) Low humidity reduces ectoparasite pressure: implications for host life history evolution. *Oikos* **97**, 223-228.

Moyer BR, Gardiner DW, Clayton DH (2002b) Impact of feather molt on ectoparasites: looks can be deceiving. *Oecologia* **131**, 203-210.

Nelson RJ, Demas GE, Klein SL, Kriegsfeld LJ (2002) *Seasonal Patterns of Stress, Immune Function and Disease* Cambridge University Press, Cambridge.

Norris K, Evans MR (2000) Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology* **11**, 19-26.

Ochsenbein AF, Fehr T, Lutz C*, et al.* (1999) Control of early viral and bacterial distribution and disease by natural antibodies. *Science* **286**, 2156-2159.

Ochsenbein AF, Zinkernagel RM (2000) Natural antibodies and complement link innate and acquired immunity. *Immunology Today* **21**, 624-630.

Padilla LR, Whiteman NK, Merkel J, Huyvaert KP, Parker PG (2006) Health assessment of seabirds on isla Genovesa, Galapagos Islands *Ornithological Monographs* (2006), Chapter 6, pages 1-12.

Parejo D, Silva N (2009) Immunity and fitness in a wild population of Eurasian kestrels Falco tinnunculus. *Naturwissenschaften* **96**, 1193-1202.

Parker PG, Whiteman NK, Miller RE (2006) Conservation medicine on the galapagos islands: Partnerships among behavioral, population, and veterinary scientists. *Auk* **123**, 625-638.

Parmentier HK, Lammers A, Hoekman JJ*, et al.* (2004) Different levels of natural antibodies in chickens divergently selected for specific antibody responses. *Developmental and Comparative Immunology* **28**, 39-49.

Pearman PB, Garner TWJ (2005) Susceptibility of Italian agile frog populations to an emerging strain of Ranavirus parallels population genetic diversity. *Ecology Letters* **8**, 401-408.

Poulin R, Marshall LJ, Spencer HG (2000a) Genetic variation and prevalence of blood parasites do not correlate among bird species. *Journal of Zoology* **252**, 381-388.

Poulin R, Marshall LJ, Spencer HG (2000b) Metazoan parasite species richness and genetic variation among freshwater fish species: cause or consequence? *International Journal for Parasitology* **30**, 697-703.

Puurtinen M, Hytonen M, Knott KE*, et al.* (2004) The effects of mating system and genetic variability on susceptibility to trematode parasites in a freshwater snail, Lymnaea stagnalis. *Evolution* **58**, 2747-2753.

Reauz B, Scope A, Hauska H, Vasicek L (1999) Comparison of haematological examination methods in birds. *Tierarztliche Praxis Ausgabe Kleintiere Heimtiere* **27**, 65-70.

Reed DH (2004) Extinction risk in fragmented habitats. *Animal Conservation* **7**, 181-191.

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. *Conservation Biology* **17**, 230-237.

Reid JM, Arcese P, Keller LF (2003) Inbreeding depresses immune response in song sparrows (Melospiza melodia): direct and inter-generational effects. *Proceedings of the Royal Society of London Series B-Biological Sciences* **270**, 2151-2157.

Reid JM, Arcese P, Keller LF*, et al.* (2007) Inbreeding effects on immune response in free-living song sparrows (Melospiza melodia). *Proceedings of the Royal Society B-Biological Sciences* **274**, 697-706.

Ross-Gillespie A, O'Riain MJ, Keller LF (2007) Viral epizootic reveals inbreeding depression in a habitually inbreeding mammal. *Evolution* **61**, 2268-2273.

Salam ST, Mir MS, Khan AR (2009) Prevalence and seasonal variation of ectoparasite load in free-range chicken of Kashmir valley. *Tropical Animal Health and Production* **41**, 1371-1376.

Sarker N, Tsudzuki M, Nishibori M, Yasue H, Yamamoto Y (2000) Cell-mediated and humoral immunity and phagocytic ability in chicken lines divergently selected for serum immunoglobulin M and G levels. *Poultry Science* **79**, 1705-1709.

Shaffer ML (1981) Minimum Population Sizes for Species Conservation. *Bioscience* **31**, 131-134.

Slate J, David P, Dodds KG*, et al.* (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* **93**, 255-265.

Smith KF, Acevedo-Whitehouse K, Pedersen AB (2009) The role of infectious diseases in biological conservation. *Animal Conservation* **12**, 1-12.

Soos C, Padilla L, Iglesias A, *et al.* (2008) Comparison of pathogens in broiler and backyard chickens on the Galapagos Islands: Implications for transmission to wildlife. *Auk* **125**, 445-455.

Spielman D, Brook BW, Briscoe DA, Frankham R (2004a) Does inbreeding and loss of genetic diversity decrease disease resistance? *Conservation Genetics* **5**, 439-448.

Spielman D, Brook BW, Frankham R (2004b) Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 15261-15264.

Stevens L, Yan GY, Pray LA (1997) Consequences of inbreeding on invertebrate host susceptibility to parasitic infection. *Evolution* **51**, 2032-2039.

Taylor SS, Jamieson IG (2007) Factors affecting the survival of founding individuals in translocated New Zealand Saddlebacks Philesturnus carunculatus. *Ibis* **149**, 783-791.

Tompkins DM, Mitchell RA, Bryant DM (2006) Hybridization increases measures of innate and cell-mediated immunity in an endangered bird species. *Journal of Animal Ecology* **75**, 559-564.

Van Riper C, Scott JM (2001) Limiting factors affecting Hawaiian native birds. In: *Evolution, ecology, conservation, and management of Hawaiian birds: a vanishing avifauna. Studies in Avian Biology* (eds. Scott JM, Conant S, Van Riper C), pp. 221-233. Cooper Ornithological Society, Camarillo, CA.

Van Riper C, Van Riper SG, Goff ML, Laird M (1986) The Epizootiology and Ecological Significance of Malaria in Hawaiian Land Birds. *Ecological Monographs* **56**, 327-344.

Van Riper C, Van Riper SG, Hansen WR (2002) Epizootiology and effect of avian pox on Hawaiian forest birds. *Auk* **119**, 929-942.

Vargas H (1987) Frequency and Effect of Pox-Like Lesions in Galapagos Mockingbirds. *Journal of Field Ornithology* **58**, 101-102.

Vargas H, Snell HL (1997) The arrival of Marek's disease to Galapagos. *Noticias de Galapagos* **58**, 4-5.

Visscher PM, Smith D, Hall SJG, Williams JA (2001) A viable herd of genetically uniform cattle - Deleterious alleles seem to have been purged in a feral strain of inbred cows. *Nature* **409**, 303-303.

Wakelin D (1996) *Immunity to Parasites. How Parasitic Infections are Controlled* Cambridge University Press, Cambridge.

Walther BA, Clayton DH (1997) Dust-ruffling: A simple method for quantifying ectoparasite loads of live birds. *Journal of Field Ornithology* **68**, 509-518.

Whiteman NK, Matson KD, Bollmer JL, Parker PG (2006) Disease ecology in the Galapagos Hawk (Buteo galapagoensis): host genetic diversity, parasite load and natural antibodies. *Proceedings of the Royal Society B-Biological Sciences* **273**, 797-804.

Whiteman NK, Parker PG (2004) Body condition and parasite load predict territory ownership in the Galapagos Hawk. *Condor* **106**, 915-921.

Wiehn J, Kopp K, Rezzonico S, Karttunen S, Jokela J (2002) Family-level covariation between parasite resistance and mating system in a hermaphroditic freshwater snail. *Evolution* **56**, 1454-1461.

Wikel SK, Alarcon-Chaidez FJ (2001) Progress toward molecular characterization of ectoparasite modulation of host immunity. *Veterinary Parasitology* **101**, 275-287.

Wikelski M, Foufopoulos J, Vargas H, Snell H (2004) Galapagos Birds and Diseases: Invasive Pathogens as Threats for Island Species. *Ecology and Society* **9**.

Zouali M (2001) Natural Antibodies. In: *Encyclopedia of Life Sciences*. John Wiley & Sons, Ltd.

Zuk M, Stoehr AM (2002) Immune defense and host life history. *American Naturalist* **160**, S9-S22.

FIGURES & TABLES

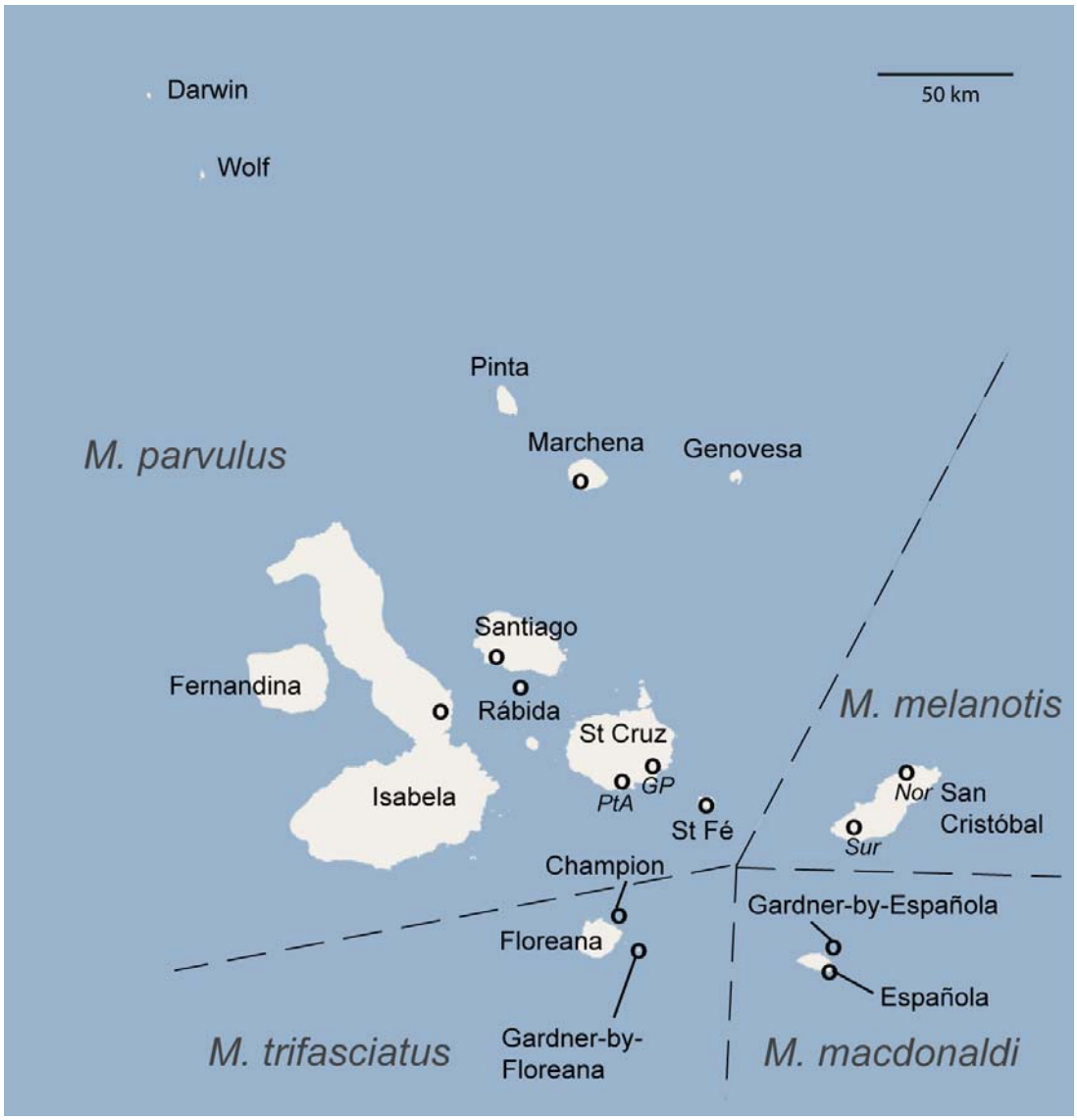

Figure 1:

Distribution of the four different mockingbird species (*Mimus* spp.) in the Galápagos archipelago with circles indicating the sites of sample collection. On St. Cruz (*PtA* and *GP*) and San Cristóbal (*Sur* and *Nor*) samples were obtained from two different locations.

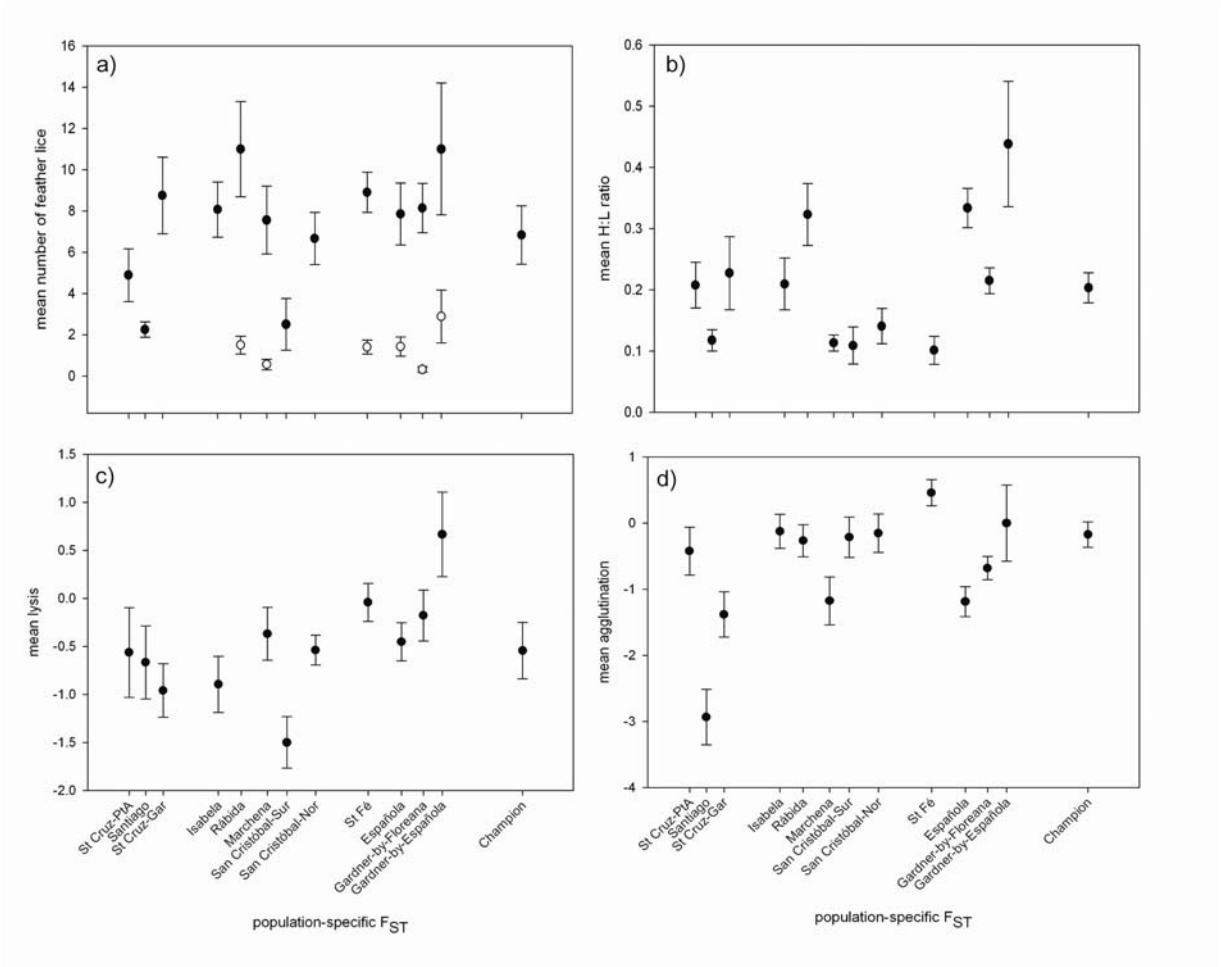

Figure 2:

The three estimates of immunocompetence and feather louse numbers shown as a function of population-specific Fst, a measure of inbreeding, with values of Fst increasing from left to right (real numbers are given in Table 1 and Figure 3). To improve visual representation, points that overlapped (on the x-axis) were slightly moved. Bars represent the standard errors of the means. a) The mean number of *Myrsidea* (closed circles) and *Brueelia* (open circles) lice per population. *Brueelia* did not occur in all populations. The total mean number of feather lice per population is shown in Table 1. b) The mean heterophil-lymphocyte (H:L) ratio of each population. c) Mean lysis and d) mean agglutination scores for each population, with values corrected for the chicken control sample.

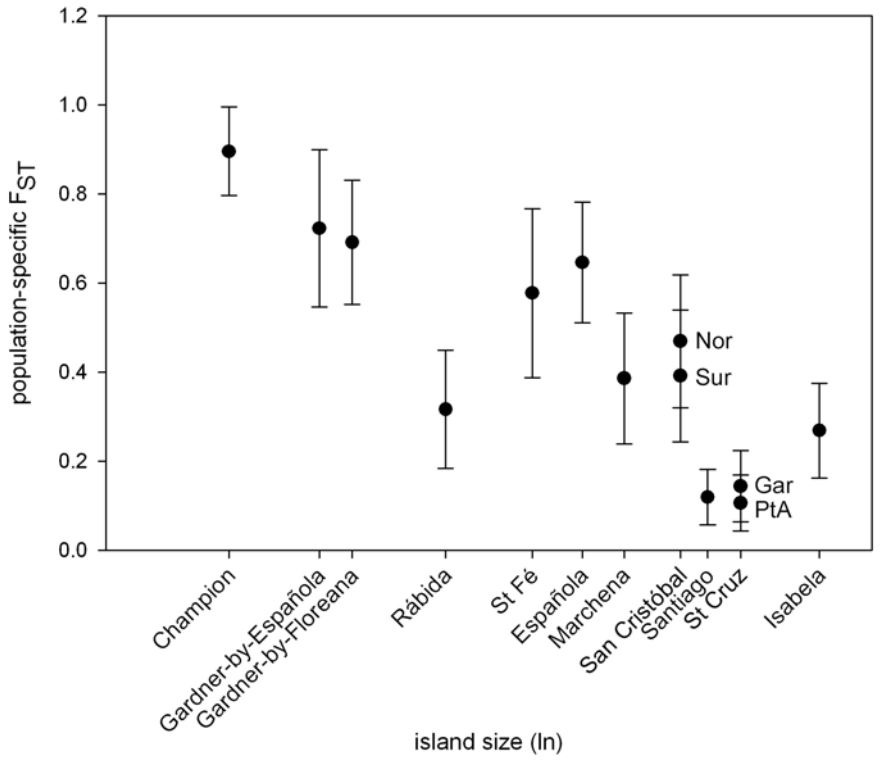

Figure 3:

Population-specific Fst as a function of the natural logarithm of island size (in ha). Points that overlapped were slightly moved to improve visual representation. The two Fst estimates for St. Cruz and San Cristóbal represent the two different sampling sites on these islands. Bars show the minimum and maximum estimates for Fst as calculated in 2mod (Ciofi *et al.*, 1999).

Table 1:

Populations studied with island size (log-scale, in hectares), estimates of genetic diversity and immunocompetence and the respective sample sizes (N, number of individual mockingbirds). Measures of genetic diversity are based on 26 microsatellite loci: NA: number of alleles, AR: allelic richness, He: expected heterozygosity, pop. Fst: population-specific Fst as calculated in 2mod (Ciofi *et al.*, 1999), temp. Fst: temporal Fst as calculated in Hoeck *et al.* (in press-b). Mean lysis and agglutination scores are given (for chicken control corrected values see Fig. 2c & d), as well as mean heterophil-lymphocyte (H:L) ratio and mean number of total feather lice encountered per individual in each population.

		genetic diversity						lysis score		agglutination score		H:L ratio		# of feather lice	
Island	**island size (ln; ha)**	**NA**	**AR**	**He**	**N**	**pop. Fst**	**temp. Fst**	**Mean**	**N**	**Mean**	**N**	**Mean**	**N**	**Mean**	**N**
Champion	2.25	32	1.18	0.06	48	0.90	0.28	3.27	24	9.33	24	0.20	62	6.83	12
Espanola	8.71	68	2.08	0.25	58	0.65	0.02	3.58	43	8.48	63	0.33	88	9.27	33
Gardner-by-Espanola	4.06	45	1.73	0.17	10	0.72	0.11	4.33	3	9.33	3	0.44	10	13.89	9
Gardner-by-Floreana	4.4	62	2.12	0.32	69	0.69	0.08	3.68	31	9.18	44	0.21	72	8.45	22
Isabela	13.04	128	3.77	0.48	32	0.27	0.02	2.95	19	9.19	16	0.21	29	8.07	14
Marchena	9.47	95	3.04	0.47	38	0.39	0.02	3.55	19	8.45	20	0.11	39	8.11	18
Rabida	6.21	96	3.29	0.51	21	0.32	0.05	.	0	9.13	15	0.32	21	12.50	10
SanCristobal-Sur	10.93	96	3.23	0.40	20	0.39	0.02	2.50	12	9.46	14	0.11	12	2.50	6
SanCristobal-Nor	10.93	79	2.78	0.37	17	0.47	0.02	3.42	13	9.31	13	0.14	17	6.67	15
Santiago	10.98	154	4.69	0.61	27	0.12	0.01	3.70	15	7.37	15	0.12	27	2.25	12
StCruz-Gar	11.5	133	4.37	0.63	22	0.14	0.01	3.17	12	8.35	17	0.23	38	8.75	16
StCruz-PtA	11.5	137	4.68	0.65	17	0.11	0.01	3.31	8	8.77	13	0.21	17	4.89	9
StFe	7.79	59	2.13	0.33	21	0.58	0.01	3.71	12	9.71	12	0.10	21	10.30	20
Total # of samples					400				211		269		453		196

Table 2:

GLMs for a) agglutination and b) lysis scores (corrected for chicken control sample), c) heterophil-lymphocyte (H:L) ratio (ln-transformed), and d) number of feather lice (transformed to the power of 0.25).

	Estimate	±SE	95%CI	t-value	d.f.	p-value
a) Agglutination						
Intercept	-1.57	0.56	-2.8 – -0.3	-2.82	1,10	
population-specific Fst	2.23	1.42	-0.9 – 5.4	1.56	1,10	0.15
temporal Fst	-2.24	4.76	-13.0 – 8.5	-0.47	1,10	0.65
b) Lysis						
Intercept	-0.91	0.35	-1.7 – -0.1	-2.64	1, 9	
population-specific Fst	1.11	0.89	-0.9 – 3.1	1.25	1, 9	0.24
temporal Fst	-1.21	2.97	-7.9 – 5.5	-0.41	1, 9	0.69
c) H:L ratio						
Intercept	-2.30	0.31	-2.9 – -1.6	-7.51	1,10	
population-specific Fst	0.47	0.79	-1.3 – 2.2	0.59	1,10	0.57
temporal Fst	0.88	2.63	-5.1 – 6.8	0.33	1,10	0.75
d) Feather lice						
Intercept	1.24	0.16	0.8 – 1.6	7.77	1,10	
population-specific Fst	0.59	0.41	-0.3 – 1.5	1.45	1,10	0.18
temporal Fst	-0.75	1.37	-3.9 – 2.4	-0.55	1,10	0.60

ADDITIONAL INFORMATION, CONCLUSIONS, AND PERSPECTIVES

The Floreana mockingbird and its conservation

Galápagos is unique and fortunate in that none of the 58 resident bird species, of which 54% are endemic, have gone extinct. This is especially surprising when one considers that oceanic avifauna are 40 times more likely to become extinct than continentals (Johnson and Stattersfield, 1990) and that Hawaii, for example, has lost 37% of its endemic bird species (Warner, 1968, Atkinson et al., 2000). However, Galápagos has also experienced dramatic, anthropogenic changes which put the native flora and fauna at risk. The Floreana mockingbird and the Mangrove finch (*Cactospiza heliobates*) are two critically endangered Galápagos endemics. Fortunately, for both species recovery plans exist and protection measures managed by the Charles Darwin Foundation and the Galápagos National Park are under way.

Among the Galápagos Islands, Floreana has sustained the greatest loss in biodiversity, due to a combination of factors related to the arrival and settlement of humans, including habitat destruction, predation and competition by introduced species, and human activity (CDF, 2008). Although some of the plant and animal species lost on Floreana cannot be recovered, the Floreana mockingbird (*Mimus trifasciatus*) still exists on the two satellite islands Champion and Gardner-by-Floreana. In 2007 we held a workshop and developed a plan under the leadership of the Charles Darwin Foundation and the Galápagos National Park Service to reintroduce *M. trifasciatus* onto Floreana in line with a whole island restoration project (CDF, 2008, CDF, 2009). The study described in Chapter 3 of this thesis covers one of the objectives described in the reintroduction plan, namely to determine the genetic composition and the genetic relationship of the two remaining mockingbird populations on Champion and Gardner. The genetic data presented serve as a baseline for future genetic monitoring of the species to determine how much genetic diversity increases or changes after reintroduction onto Floreana.

Previous to this work, loss of genetic diversity had been hypothesized in the two satellite populations due to isolation and inbreeding (Grant *et al.*, 2000). Estimates on the loss of genetic diversity, however, were based on theoretical calculations only and had not been supported by genetic data. Furthermore, a former connection between the three populations through the intermediate population on Floreana had only been speculated,

and historic and extant differentiation between the populations was unknown. The results presented in Chapter 3 are concordant with the hypothesis that gene flow once occurred between the three populations and that the current Champion and Gardner populations show high genetic differentiation due to drift, inbreeding and isolation in the recent past. Genetic variation has been lost in the Floreana mockingbird due to extinction of the Floreana population, as exemplified by the alleles detected in the two Floreana specimens but absent in the Champion and Gardner populations. However, part of the genetic variation once present on Floreana has persisted in both satellite populations, rendering them both important for reintroduction and suggesting a mixing of individuals from both islets to maximize genetic diversity in the population to be reintroduced on Floreana.

Neutral genetic markers are thought to be poor predictors of the evolutionary potential of populations because they do not reveal information about adaptive response to altered environmental conditions (see references in Ashley *et al.*, 2003). However, here I did not intend to quantify potential adaptation to the isolated environment on the satellite islands but rather confirm the hypothesized former similarity between the two satellite populations and their former connection to Floreana. Gene flow from Floreana back to the satellite islands, especially to the very closely located Champion, is likely to occur once a large enough population has established itself on Floreana. This could, hence, in the long-term possibly re-establish a migration-drift equilibrium between the populations. Whether the two populations have adapted differently to their local environment and whether the re-establishment of gene flow could hence disrupt such adaptation, cannot be answered with the data presented in this thesis. Also, it is unknown whether the two contemporary populations suffer from inbreeding depression in individual fitness due to the presently low genetic diversity. At least the immunological data presented in Chapter 4 do not suggest a relationship between inbreeding levels and one aspect of population fitness. However, as inbreeding depression is a well-known phenomenon in wild animal and plant populations (Keller and Waller, 2002), and, on the other hand, gene flow or interbreeding have been shown to increase additive genetic variance (e.g. Grant and Grant, 2008) and lead to heterosis (Westemeier *et al.*, 1998, Madsen *et al.*, 1999, Hogg *et al*., 2006, Whitlock *et al*., 2000), it is reasonable to assume

that mixing individuals from both satellite populations will benefit the reintroduced population on Floreana. Even without direct evidence of inbreeding depression the species is likely to benefit from enhanced genetic diversity to increase fitness and evolutionary adaptive potential, as it has been shown in other species (e.g. Hogg *et al.*, 2006, Fredrickson *et al*., 2007, Ingvarsson, 2001). Possible effects of outbreeding depression can, of course, not be ruled out completely, but are considered unlikely as the two satellite populations live in a very similar environment and do not have a long, independent evolutionary history (Chapter 3). Furthermore, introductions of individuals from related populations into very small endangered populations with low fitness more often result in hybrid vigor rather than outbreeding depression (e.g. Westemeier et al., 1998, Fredrickson et al., 2007, Madsen et al., 1999, Madsen et al., 2004).

Besides the planned establishment of a third population on Floreana, current and future conservation and management actions should focus on conserving the two satellite populations, especially by averting the introduction of new predators or diseases to the satellite islets.

Despite the potentially beneficial genetic effects of gene flow that might follow the reintroduction to Floreana, migration of individuals between Floreana and its two satellite populations might also carry pathogen-related risks. Disease agents in both the source mockingbird populations as well as in the wild birds and poultry to which these birds will be exposed on Floreana could lead to disease-related morbidity or mortality. Disease risks have long been appreciated in reintroduction plans, but few have included studies and measures to minimize these risks (Cunningham, 1996). Within the mockingbird reintroduction plan, the determination of the current disease threats and risks prior to the reintroduction of the mockingbirds back onto Floreana was identified as a top priority item (CDF, 2008). The immunological results gained in Chapter 4 suggest that the mockingbirds show quite a strong innate immunocompetence, hence first line of defense against invading pathogens. From these results, however, it cannot be inferred whether the mockingbirds may also be equipped with a strong adaptive immunity. We do not know how the Floreana mockingbirds might cope with new, introduced diseases, such as pathogens found in domestic chickens and wild passerines on Floreana (Deem *et al.*, in

prep.), as well as in chickens on other Galápagos islands (Gottdenker *et al*., 2005, Soos *et al*., 2008). The possibility of transmission of infectious agents from chicken farms to the mockingbirds is therefore of special concern. If any of these disease agents are present on Floreana and not present on the satellite islands, they will have great potential of causing high mortality in the reintroduced birds since they will have little to no immunity to these pathogens. Therefore, a systematic disease survey in domestic and wild birds on Floreana has been conducted by colleagues from the University of Missouri and St. Louis Zoo, USA (Deem *et al.*, in prep.). The analyses of plasma samples from Champion and Gardner to investigate the presence of pathogens on these satellite islets are currently underway. This health survey will inform the reintroduction project on measures of how to best avoid disease risks in the Floreana mockingbirds.

The former mockingbird populations on Baltra and North Seymour islands

In Chapter 2, I included the analysis of historic samples from Baltra Island where mockingbirds went extinct some 60-70 years ago following the establishment of a military base on the island (Curry, 1986), as information on the former population's genetic relationship to its neighbouring islands might be helpful in the future. As expected, mockingbirds on Baltra were most closely related to the Santa Cruz population, followed by Santiago. In case reintroduction plans are ever established for Baltra like they are already underway for the Floreana mockingbird, these results suggest that individuals from Santa Cruz should be taken for reintroduction onto Baltra.

Reintroduction of mockingbirds following habitat restoration should also potentially be considered for North Seymour Island. When visiting the island in 2007, we were surprised not to detect a single mockingbird after intense searches that covered the entire island, and despite the general believe that mockingbirds inhabit the island (Felipe Cruz, personal comm.). That mockingbirds used to occur there but disappeared in the past few years was confirmed by various Galápagos naturalist guides who visit North Seymour on a regular basis. They mentioned the introduction of black rats as a potential cause for the mockingbirds' disappearance. I analyzed four historic specimens from the California Academy of Sciences collection that were collected on North Seymour in 1899. However, these specimens were omitted from all analyses presented in Chapter 2 due to the small sample size. Based on the genetic results from these 4 individuals, it seems that the former North Seymour population was, not surprisingly, most closely related to the former Baltra population and the Santa Cruz mockingbirds. The latter would hence serve best as a potential source population for reintroduction. Given the close proximity of Baltra and North Seymour to Santa Cruz, however, mockingbirds might be able to recolonize the islands without any reintroduction efforts once the causes of decline have been removed from these places. Further investigations on the potential causes for the absence of mockingbirds on Baltra and North Seymour and monitoring of passerines on these two islands are therefore recommended.

Co-evolution and biogeography of Galápagos mockingbirds and their ectoparasites

An interesting new project emerged through the extensive ectoparasite samples collected from all four species of mockingbirds during the course of this thesis (Chapter 4). Dr. Vincent Smith and Dr. Jan Stefka from the Natural History Museum, UK, have started a study that examines the co-evolutionary history of multiple ectoparasite lineages with the mockingbirds. By reconstructing the evolutionary history of Galápagos mockingbird lice and mites, they are testing assumptions about the monophyly and colonization of mockingbirds across the Galápagos. Using both morphological and molecular genetic techniques, they compare and contrast the genetic structure of mockingbird ectoparasites at six different scales (between and within host species, islands, and parasite groups) for the parasitic lice and mites we collected on 11 different islands. Each widespread parasite lineage forms an ecological replicate to test the relative contributions of host diversification and geography to patterns of parasite genetic differentiation and speciation. Hence, this system provides a framework to improve the general understanding on the correlates of parasite diversification, their mode of speciation and the evolution of host specialization. Furthermore, the data will reveal information on how louse-vectored diseases may be spread and, therefore, help inform conservation plans.

REFERENCES

ASHLEY, M. V., WILLSON, M. F., PERGAMS, O. R. W., O'DOWD, D. J., GENDE, S. M. & BROWN, J. S. (2003) Evolutionarily enlightened management. *Biological Conservation,* 111**,** 115-123.

ATKINSON, C. T., DUSEK, R. J., WOODS, K. L. & IKO, W. M. (2000) Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases,* 36**,** 197-204.

CDF (2008) *The Reintroduction of the Floreana Mockingbird to its Island of Origin,* Puerto Ayora, Galapagos, Charles Darwin Foundation & Galapagos National Park Service.

CDF (2009) *Project Floreana 2009-2013: An integrated approach,* Puerto Ayora, Galapagos.

CUNNINGHAM, A. A. (1996) Disease risks of wildlife translocations. *Conservation Biology,* 10**,** 349-353.

CURRY, R. L. (1986) Whatever happened to the Floreana mockingbird? *Noticias de Galapagos,* 43**,** 13-15.

DEEM, S. L., HOECK, P. E. A., CRUZ, M., MERKEL, J. & PARKER, P. G. (in prep.) Disease risks associated with the re-introduction of the Floreana mockingbird (Mimus trifasciatus) to its island of origin in the Galapagos.

FREDRICKSON, R. J., SIMINSKI, P., WOOLF, M. & HEDRICK, P. W. (2007) Genetic rescue and inbreeding depression in Mexican wolves. *Proceedings of the Royal Society B-Biological Sciences,* 274**,** 2365-2371.

GOTTDENKER, N. L., WALSH, T., VARGAS, H., MERKEL, J., JIMENEZ, G. U., MILLER, R. E., DAILEY, M. & PARKER, P. G. (2005) Assessing the risks of introduced chickens and their pathogens to native birds in the Galapagos Archipelago. *Biological Conservation,* 126**,** 429-439.

GRANT, P. R., CURRY, R. L. & GRANT, B. R. (2000) A remnant population of the Floreana mockingbird on Champion island, Galapagos. *Biological Conservation,* 92**,** 285-290.

GRANT, P. R. & GRANT, B. R. (2008) *How and Why Species Multiply. The radiation of Darwin's Finches,* Princeton, NJ, Princeton University Press.

HOGG, J. T., FORBES, S. H., STEELE, B. M. & LUIKART, G. (2006) Genetic rescue of an insular population of large mammals. *Proceedings of the Royal Society B-Biological Sciences,* 273**,** 1491-1499.

INGVARSSON, P. K. (2001) Restoration of genetic variation lost - The genetic rescue hypothesis. *Trends in Ecology & Evolution,* 16**,** 62-63.

JOHNSON, T. H. & STATTERSFIELD, A. J. (1990) A global review of island endemic birds. *Ibis,* 132**,** 167-180.

KELLER, L. F. & WALLER, D. M. (2002) Inbreeding effects in wild populations. *Trends in Ecology & Evolution,* 17**,** 230-241.

MADSEN, T., SHINE, R., OLSSON, M. & WITTZELL, H. (1999) Conservation biology - Restoration of an inbred adder population. *Nature,* 402**,** 34-35.

MADSEN, T., UJVARI, B. & OLSSON, M. (2004) Novel genes continue to enhance population growth in adders (Vipera berus). *Biological Conservation,* 120**,** 145-147.

SOOS, C., PADILLA, L., IGLESIAS, A., GOTTDENKER, N., BEDON, M. C., RIOS, A. & PARKER, P. G. (2008) Comparison of pathogens in broiler and backyard chickens on the Galapagos Islands: Implications for transmission to wildlife. *Auk,* 125**,** 445-455.

WARNER, R. E. (1968) Role of Introduced Diseases in Extinction of Endemic Hawaiian Avifauna. *Condor,* 70**,** 101-&.

WESTEMEIER, R. L., BRAWN, J. D., SIMPSON, S. A., ESKER, T. L., JANSEN, R. W., WALK, J. W., KERSHNER, E. L., BOUZAT, J. L. & PAIGE, K. N. (1998) Tracking the long-term decline and recovery of an isolated population. *Science,* 282**,** 1695-1698.

WHITLOCK, M. C., INGVARSSON, P. K. & HATFIELD, T. (2000) Local drift load and the heterosis of interconnected populations. *Heredity,* 84**,** 452-457.

SUMMARY

One of the four endemic, allopatrically living mockingbird species in the Galápagos, the Floreana mockingbird (*Mimus trifasciatus*), became extinct on the large island of Floreana approx. 130 years ago. Today, only two small populations survive on two isolated satellite islands. Such small and isolated populations are not only more susceptible to demographic and environmental stochastic events, but may also suffer from inbreeding. Genetic drift and inbreeding reduce genetic diversity and increase differentiation among populations. Additionally, introduced pathogens may put isolated populations at risk because of their unadapted immune systems. Reduced genetic diversity can compromise the evolvability of small populations and, together with higher susceptibility to disease, threaten their survival and increase the extinction risk.

The disappearance of the large and interjacent population of *M. trifasciatus* on Floreana probably not only led to a dramatic decline in the total number of individuals, but may also have disrupted gene flow between the two remaining satellite populations, resulting in genetic isolation, differentiation and loss of genetic variation. To secure the long-term survival of this historically important species, reintroduction back onto Floreana is planned. A key question raised by the recovery plan is whether the two populations have been evolving independently and whether they are inbred. To answer these questions, I assessed levels of genetic diversity and differentiation in *M. trifasciatus* and compared them to the ones found in other Galápagos mockingbird populations and species that did not experience such dramatic population size loss and isolation. I tested for signs of drift and gene flow and used historic samples to determine change in genetic diversity over the last century. Furthermore, I assessed three measures of the birds' innate immune system and ectoparasite load to study the relationship between inbreeding and immunocompetence.

In **Chapter 1** I described the development of a set of polymorphic microsatellite markers that were isolated from *Mimus parvulus*, tested for cross-species amplification in the other three mockingbird species, and designed in the light of work with historic, degraded DNA. These markers formed the basis for the genetic analyses described in the other chapters.

To study the effects of limited population size, genetic drift and gene flow on genetic diversity, I assessed genetic diversity within and differentiation among 19 mockingbird populations on 15 Galápagos islands, covering all four endemic species. Historic specimens allowed me to determine the genetic change over the last century and to estimate effective population sizes (*Ne*). The results in **Chapter 2** showed that island size serves as a good predictor for levels of genetic diversity and effective population size. Although archipelago-wide genetic diversity did not change significantly over the last century, genetic drift was pronounced in small populations, such as the two *M. trifasciatus* populations, where it led to substantial variation in allele frequencies over time and loss of genetic diversity. The clear pattern of genetically distinct populations reflects the effects of genetic drift and suggests that Galápagos mockingbirds are evolving in relative isolation.

In **Chapter 3** I integrated evolutionary thinking and conservation practice using coalescent analyses and genetic data from contemporary and historic samples to estimate divergence times between the two remaining *M. trifasciatus* populations and assess levels of genetic change. Two samples collected by Darwin and Robert Fitzroy on Floreana in 1835 allowed me to compare the genetic pattern of the former Floreana population with the one of the two contemporary satellite populations. The genetic analyses revealed substantial differentiation between the two extant populations, but the coalescence-based modelling did not indicate long, independent evolutionary histories. Although one of the satellite populations is highly inbred, both contemporary populations still harbor unique alleles once present on Floreana, suggesting that birds from both islets should be used to establish a single, mixed population on Floreana and maximize genetic diversity.

Because inbreeding may impair an individual's immune system and render it more susceptible to disease, in **Chapter 4** I tested the relationship between levels of genetic diversity and three different measures of innate immunity in 13 different mockingbird populations, again covering the range of all four species. I used a larger set of microsatellite loci and genetic data from historic and contemporary samples to calculate short-term and long-term inbreeding and compared these inbreeding estimates with estimates of immunocompetence. I detected no significant effect of either measure of inbreeding on natural antibody or complement enzyme titres, heterophil-lymphocyte ratio or mean number of feather lice. The data therefore do not support a link between inbreeding and innate immune response or ectoparasite load in the mockingbirds. However, the relatively high levels of natural antibodies and complement enzymes suggest that the mockingbirds have a strong first line of defense against invading pathogens.

In conclusion, these findings suggest an important role of genetic drift in the shaping of the genetic structure of different mockingbird populations and species in the Galápagos. The two Floreana mockingbird populations experienced the most substantial genetic change during the past century and are genetically isolated today. However, they both contain valuable genetic variation for the re-establishment of a third population on Floreana. This study shows how genetic data and the analysis of historic specimens provide an insight into the genetic structure and evolutionary forces in different-sized island populations, and how evolutionary thinking can have an immediate relevance in the design of effective conservation strategies for an endangered species.

ZUSAMMENFASSUNG

Die Spottdrosseln von Galápagos haben sich ihren Platz in der Wissenschaft schon im 19. Jahrhundert gesichert. Durch ihr unterschiedliches Aussehen auf verschiedenen Inseln haben sie Darwin zu seiner berühmten Theorie der natürlichen Selektion inspiriert. In Galápagos gibt es heute vier endemische, allopatrisch lebende Spottdrosselarten der Gattung *Mimus*. Als Darwin Galápagos 1835 besuchte, konnte er noch Spottdrosseln der Art *Mimus trifasciatus* auf der Insel Floreana beobachten und für seine Tierkollektion mitnehmen. Heute findet man auf Floreana keine Spottdrosseln mehr. Es gibt nur noch zwei kleine Restpopulationen (ca. 20-50 und 200-500 Individuen) von *M. trifasciatus* auf zwei Satelliteninseln vor Floreana und die Art ist heute von der IUCN als kritisch gefährdet eingestuft. Die menschliche Besiedlung von Floreana und die damit einhergehende Lebensraumzerstörung, sowie die Einfuhr von Katzen und Ratten sind sehr wahrscheinlich der Grund für das Verschwinden der Vögel auf dieser Insel.

Kleine, isoliert lebende Lebensgemeinschaften, wie sie auf diesen beiden Satelliteninseln vorkommen, sind nicht nur anfälliger für demographische und umweltbedingte Schwankungen, sondern leiden auch unter einem schnelleren Verlust an genetischer Diversität. Dafür sind Prozesse wie die genetische Drift (die zufällige Veränderung der Allelfrequenzen), aber auch das vermehrte Vorkommen von Inzucht (die Verpaarung verwandter Individuen) verantwortlich, da der Austausch von genetischer Variabilität durch die Migration von Individuen zwischen Populationen in isolierten Lebensräumen nicht mehr gewährleistet ist. Dies führt auch zu einer Zunahme der genetischen Unterschiede zwischen Populationen. Isolierte Lebensgemeinschaften sind zudem besonders anfällig für fremdartige Krankheitserreger, da ihr Immunsystem nicht auf diese neuen Erreger eingestellt ist. Sowohl der Verlust an genetischer Diversität durch Drift und Inzucht, wie auch die erhöhte Krankheitsanfälligkeit, können erheblich zur Bedrohung und zum Aussterben seltener Arten beitragen und erfordern daher für den Naturschutz und den Erhalt der Biodiversität besondere Beachtung. Im Falle der Floreana Spottdrossel wurde angenommen, dass mit dem Verschwinden der Population auf Floreana vor ca. 130 Jahren nicht nur die meisten Individuen von *M. trifasciatus* verschwunden sind, sondern auch die einzige Verbindung zwischen den zwei verbleibenden Satellitenpopulationen, was zu einem Abbruch des Genflusses geführt haben könnte. Solch vollständige genetische Isolation der beiden Kleinpopulationen hätte

somit Inzucht und evtl. auch eine Einbusse an evolutionärem Anpassungspotenzial zur Folge, was die Aussterbewahrscheinlichkeit erhöhen würde. Um diese historisch wichtige und charismatische Art langfristig zu schützen, ist eine Wiederansiedlung auf Floreana geplant.

Ziel dieser Studie war es, die genetische Struktur und die Krankheitsanfälligkeit der beiden bedrohten *M. trifasciatus* Populationen genauer zu untersuchen, indem sie mit verschiedenen Populationen anderer Spottdrosselarten in den Galápagos verglichen werden, die keine so dramatische Populationseinbusse oder Isolierung erfahren haben. Haben durch das Verschwinden der grossen Population auf Floreana der Inzuchtgrad und die genetischen Unterschiede zwischen den zwei verbleibenden Populationen wie erwartet zugenommen? Sind die Tiere ingezüchtet, und welche eignen sich am besten für die geplante Wiederansiedlung? Um diese und andere Fragen zu beantworten, habe ich die genetische Diversität von 19 verschiedenen Spottdrosselpopulationen auf 15 Inseln untersucht und sie mit der von *M. trifasciatus* verglichen. Spottdrosselbälge, die während den grossen Forschungsexpeditionen im 19. Jahrhundert gesammelt und heute weltweit in verschiedenen Museen aufbewahrt sind, haben es mir zudem ermöglicht, Veränderungen in der genetischen Variabilität von *M. trifasciatus* vor und nach dem Abbruch des Genflusses zu bestimmen (sozusagen den „Originalzustand“ zu verstehen) und mit dem genetischen Wandel anderer, unterschiedlich grosser Spottdrosselpopulationen zu vergleichen. Um die Beziehung zwischen Inzuchtgrad und Krankheitsanfälligkeit zu untersuchen, habe ich ausserdem drei Komponenten des angeborenen Immunsystems und den Federparasitenbefall der Vögel untersucht.

Die genetischen Analysen der heutigen und hundertjährigen Proben haben gezeigt, dass es sehr grosse Unterschiede in der genetischen Struktur und dem Inzuchtgrad zwischen unterschiedlich grossen Populationen gibt. Wie erwartet, korreliert die genetische Diversität einer Population stark mit der Inselgrösse und nehmen die genetischen Unterschiede mit der geographischen Distanz zwischen den Populationen zu. Die Effekte der genetischen Drift sind stark von der Populationsgrösse abhängig und haben in den kleinen Populationen zu grossen genetischen Veränderungen über die letzten hundert Jahre geführt. Dies ist vor allem in den beiden *M. trifasciatus* Populationen der Fall, von denen die kleinere stark ingezüchtet ist. Generell lassen die

Daten darauf schliessen, dass Genfluss (Migration von Individuen) zwischen verschiedenen Inseln bei den Spottdrosseln in Galápagos selten ist, und dass kein genetischer Ausstausch zwischen den verbleibenden *M. trifasciatus* Populationen stattfindet. Obwohl die beiden Populationen heute sehr unterschiedliche genetische Muster zeigen, beherbergen sie beide einzigartige genetische Information, die damals in der grossen Population auf Floreana vorkam. Die immunologischen Analysen zeigen nicht wie erwartet eine negative Beziehung zwischen Inzuchtgrad und Immunokompetenz verschiedener Populationen, sondern deuten darauf hin, dass die Spottdrosseln von Galápagos unabhängig von ihrer genetischen Diversität eine relativ gute angeborene Immunabwehr haben. Ob dies aber auch für das erworbene Immunsystem zutrifft, und wie die Vögel auf eingeführte Krankheiten reagieren würden, bleibt Gegenstand künftiger Untersuchungen.

Zusammenfassend lässt sich sagen, dass Drift deutliche Auswirkungen auf die genetische Struktur verschiedener Spottdrosselpopulationen hat und vor allem in kleinen Populationen sehr ausgeprägt ist. Während die kleinere der beiden *M. trifasciatus* Populationen sehr ingezüchtet ist, enthält die zweite Population noch erstaunlich viel genetische Diversität. Da aber auf beiden Satelliteninseln einmalige genetische Variation vorhanden ist, sollten trotzdem Individuen von beiden Populationen für das Wiederansiedlungsprojekt auf Floreana geommen werden, um die genetische Diversität und somit das Evolutionspotential der neuen Population so gut als möglich zu maximieren. Diese Studie führt vor, wie genetische Daten unter Einbezug von historischen Proben nicht nur einen Einblick in die Struktur, die genetischen Veränderungen und somit evolutionären Kräfte in verschieden grossen Inselpopulationen geben können, sondern dass diese auch direkt für Schutzmassnahmen von bedrohten Arten von Nutzen sein können.

RESUMEN

Los cucuves de Galápagos, debido a sus diferencias morfológicas, ya jugaron un papel importante para la ciencia en el siglo XIX, inspirando a Charles Darwin para el desarrollo de su famosa teoría de la selección natural. Actualmente existen cuatro especies de cucuves endémicas (género *Mimus*) de las Islas Galápagos y distribuidas por 16 islas. Sin embargo cuando Charles Darwin viajaba por Galápagos en 1835, todavía podía observarse otra población en la isla Floreana, hoy extinta. El Cucuve de Floreana *Mimus trifasciatus* desapareció de Floreana aproximadamente en 1880, probablemente debido a la pérdida y alteración del hábitat así como a los efectos negativos derivados de la introducción de gatos y ratas, quedando relegada en la actualidad a dos islotes adyacentes a Floreana, Champion y Gardner-por-Floreana. Dado su reducido tamaño de población, cifrado en unos 150-500 individuos, y su aislamiento, esta especie se encuentra clasificada como en peligro crítico (IUCN).

Poblaciones pequeñas y aisladas, como las de Champion y Gardner, no sólo son más vulnerables a variaciones estocásticas demográficas y ambientales, sino que también son más susceptibles a sufrir una pérdida acelerada de variabilidad genética, causada por la deriva genética (el cambio aleatorio en la frecuencias de alelos) y la endogamia, así como por la interrupción del flujo genético (migración) entre poblaciones, pudiendo además aumentar la diferenciación genética entre ellas. Asimismo, las poblaciones pequeñas y aisladas son más vulnerables a la introducción de nuevos patógenos, debido a una pérdida en la capacidad de adaptación de su sistema inmunológico. Dicha pérdida de diversidad genética, así como una mayor vulnerabilidad a enfermedades, pueden tener consecuencias negativas para la viabilidad poblacional y la evolución adaptativa de especies raras y amenazadas, contribuyendo así a incrementar los riesgos de extinción. En el caso del Cucuve de Floreana, con la extinción en Floreana no sólo desapareció la mayor población de esta especie, sino también la única conexión entre las dos pequeñas poblaciones satélites, lo que probablemente resultó en la interrupción del intercambio genético entre las mismas, hace aproximadamente unos 130 años. Este aislamiento genético de las dos poblaciones ha podido tener como resultado un incremento de la endogamia y la reducción del potencial adaptativo de la especie. Por lo tanto el restablecimiento de una nueva población en Floreana, a partir de la reintroducción de

ejemplares, se presenta como una estrategia válida de cara a incrementar las probabilidades de persistencia de esta especie amenazada.

El objetivo de este estudio fue la investigación de la estructura genética y susceptibilidad ante enfermedades de las dos poblaciones actuales de *M. trifasciatus*, mediante la comparación con otras especies y poblaciones de cucuves de Galápagos que no han sufrido una disminución y aislamiento poblacionales tan severos. ¿Se ha incrementado el grado de endogamia y la diferenciación genética entre las dos poblaciones remanentes a causa de la desaparición de la especie en Floreana? ¿Cuál fue la estructura genética de la población en Floreana y cuál de las dos poblaciones satélites actuales debería ser usada para la reintroducción?

Para contestar a estas y otras preguntas, investigué la diversidad genética de 19 poblaciones de cucuves en 15 islas diferentes y la comparé con la del Cucuve de Floreana. Paralelamente a la investigación de la diversidad genética de las poblaciones modernas, también examiné especimenes de cucuves del siglo XIX depositados en diversos museos. Esto me permitió cuantificar el cambio en la estructura genética entre poblaciones históricas y contemporáneas durante los últimos cien años y determinar los cambios ocurridos desde que se interrumpió el flujo genético entre las poblaciones de *M. trifasciatus*. También investigué la relación entre el nivel de endogamia y la inmunocompetencia (propensión a enfermedades) de los cucuves, midiendo tres componentes del sistema inmunológico innato y la carga parasitaria por ectoparásitos.

Los análisis de las muestras modernas e históricas mostraron que hay diferencias significativas en los niveles de diversidad genética y diferenciación entre poblaciones de diferente tamaño. La variabilidad genética se correlacionó positivamente con la superficie total de la isla y con el tamaño efectivo de la población (Ne). Asimismo, las diferencias genéticas entre poblaciones se incrementaron en relación a la distancia geográfica entre las islas. Los efectos de la deriva genética dependen mucho del tamaño de la población y han causado cambios pronunciados en las poblaciones pequeñas en el último siglo, principalmente en las poblaciones de *M. trifasciatus* existiendo un entrecruzamiento en una de ellas (Champion). De manera general los resultados demuestran que el flujo genético entre las islas es escaso para los cucuves de Galápagos, no existiendo actualmente un intercambio genético entre las dos poblaciones de *M. trifasciatus*, las

cuales muestran además una diferenciación genética entre sí. Sin embargo, el análisis de dos muestras históricas de Floreana, indica que ambas poblaciones remanentes todavía representan genéticamente a la población extinta de Floreana y que la divergencia genética entre ellas es reciente. Los resultados inmunológicos no mostraron ninguna relación entre el nivel de endogamia y la inmunocompetencia en las diferentes poblaciones de cucuves, sugiriendo no obstante que los cucuves de Galápagos, independientemente de su grado de diversidad genética, todavía presentan una buena capacidad de defensa inmunológica innata. Si esto también ocurre para el sistema inmunológico adquirido, y cómo los cucuves se adaptarían a nuevas enfermedades introducidas es motivo para futuras investigaciones.

En resumen, los resultados demuestran que la deriva genética tiene efectos pronunciados en la estructura genética de las diferentes poblaciones y especies de cucuves de Galápagos. Aunque la población en Champion muestra un alto grado de endogamia, mientras que la de Gardner todavía muestra niveles de diversidad sorprendentemente altos, ambas poblaciones presentan una diversidad única encontrándose genéticamente aisladas hoy en día. Por ello, individuos de los dos islotes deberían ser usados para una futura reintroducción en Floreana, con el fin de maximizar la variabilidad genética y el potencial evolutivo de esta nueva población. Este estudio demuestra como la información genética, obtenida a partir de muestras históricas y contemporáneas de una especie, puede ser usada para determinar la estructura y los cambios genéticos en poblaciones de diferentes tamaños, y la utilidad que ello tiene para el desarrollo de estrategias adecuadas de conservación de especies amenazadas.

MOCKINGBIRD CENSUS METHODOLOGY

established for the two remaining Floreana mockingbird (*Mimus trifasciatus*) populations

Paquita E. A. Hoeck & Herbert Biebach

Background

The Floreana mockingbird (*Mimus trifasciatus*) is a critically endangered bird species that today only occurs on two small satellite islands, Champion and Gardner-by-Floreana, after extinction of the main population on Floreana Island in the late 19th century. The combined population size on the two satellite islands was believed to number between 80 and 260 individuals (Harris, 1973; Jiménez-Uzcátegui, 2006 and references therein; Vargas, 1996).

In March 2007, a workshop was held in Puerto Ayora, Galápagos, to plan the reintroduction of *M. trifasciatus* onto Floreana (CDF, 2008) and secure the long-term survival of this species, in line with a whole island restoration project for Floreana Island (CDF, 2009). During the workshop, it was established that for the proper planning of the reintroduction, the two remaining populations on Champion and Gardner needed to be monitored carefully, and more precise estimates of population size needed to be obtained. Ideally, the two populations should be monitored and counted twice a year. Counting and banding (for individual identification of the birds) are crucial prerequisites to gain more information about population size and demography of this species.

Problems with counting mockingbirds in Galápagos

The four endemic mockingbird species in Galápagos differ in their behavior from most other birds/animals. Having evolved in a predator-poor environment, like other Galápagos endemics, the mockingbirds are extraordinarily inquisitive and tame, approach the observer from a far distance to a few meters and become easily visible. Thus, standard methods to estimate population size, such as distance sampling, cannot be applied because a positive bias in estimated density can be expected if animals are attracted to the observer (Buckland *et al.*, 1993).

The birds' tameness and curiosity is particularly pronounced in the Floreana mockingbird. Its home range, Champion and Gardner, lie within the Galápagos National Park boundary. The two islets have never had a visitor site and are only very rarely

visited by park rangers or scientists. We worked on Champion and Gardner from 2006-2009 when we visited each islet for 2-8 days each year to catch mockingbirds, band and count them. Upon landing on the islets, we were immediately approached and followed by mockingbirds. Therefore, a method to estimate their population sizes has to take this special behavior into account in order to achieve reliable and reproducible estimates. The census method outlined below is based on the principles of the widely used capture-mark-resighting method (Seber, 1982) and was modified to account for the species-specific characteristics of the Floreana mockingbird.

Census based on capture-mark-resighting

To follow the size of a population repeatedly over years, the effort and duration of the census method becomes an important criterion. The census methodology used here relies on the establishment of a known group of color-banded mockingbirds (capture/mark, M), i.e. individuals known to be alive, and their resighting (recapture, R). It is, therefore, relatively time consuming initially, but once this group of banded birds exists, the effort can be reduced.

The basic principle of the capture-mark-resighting approach is that all (visible) mockingbirds (C) are counted in a certain area where a previously established, known number of banded individuals live (M). The number of sighted banded birds (R) in relation to sighted unbanded birds (U) during a census walk then allows the estimation of the total number of birds (N) living in the area covered by the census transects.

Specifically, R/M=C/N, where C is the number of banded (R) plus unbanded (U) birds (C=R+U) counted during the census. Hence, the total estimated number of birds in the area is N=MC/R.

Preparatory measures prior to the census

In order to establish a group of marked individuals known to be alive (M), prior to the census, as many birds as possible should be caught and banded. Mockingbirds are best captured in potter traps with a piece of banana, some water, or a piece of yellow or red plastic. We banded mockingbirds on Champion and Gardner with one numbered aluminum and two colored plastic bands for individual identification.

For subsequent censi of a population where some individuals have previously been banded, as is now the case for the Gardner and Champion populations, a new group of known banded birds (M) needs to be established each time before a new census is started. Depending on the time that has passed since the last banding and counting of birds, mortality or emigration might have reduced the number of color-banded individuals. A new color-banded group of known size can be established by banding new birds (which will compensate for the loss of banded birds) and by searching the area for previously banded birds. All these individuals, newly banded or seen just prior to the census and hence known to be alive still, will comprise the new color-banded group (M).

1. Census on Champion

Because Champion is an easily accessible, small islets of only 9.5 ha and low elevation (0-50 metres a.s.l.; see Fig 1), the entire island can be covered and searched for mockingbirds quite easily within only a few days. A previous extensive study on the Champion mockingbird population revealed fluctuations in size between 24-53 individuals and 8-12 different territories on the island (Grant *et al.*, 2000). We started banding mockingbirds on Champion in December 2006 and were able to catch all individuals except for one bird. Hence, on Champion it is possible to obtain a total population count by assuring the screening of the entire island and counting/identifying all the mockingbirds present. As the precision of such a total count is highest when all mockingbirds can be individually identified, the aim of our visits to Champion from 2006-2009 was to catch and band all unbanded birds and determine which birds banded in previous years were still alive, hence allowing the calculation of survival rates. We estimated survival to be 85% from late 2006 to early 2008, and 69% from 2008 to 2009 (see Table 1).

However, if banding of (nearly) all individuals on Champion is not possible every year, the capture-mark-resighting method to estimate population size (as performed on Gardner, see below) should also be used on Champion

Fig 1: Aerial view of Champion Islet.

Total count Champion			
Individuals	**Dec. '06**	**Jan. '08**	**Feb. '09**
Banded	19	44	46
Unbanded	1	1	1
Total	20	45	47
Survival rate	-	85%	69%

Table 1: Size of the Champion mockingbird population between 2006 and 2009 and survival rate.

2. Census on Gardner

Gardner is about nine times larger than Champion (81.2 ha) and very difficult to access because of the steep slopes and crater that form most of the island (Fig 2). There is only a limited flat area ("plateau") in the north-east of the island (Fig 2 & 3), approx. 13 ha in size (GoogleMaps), and probably forming about a third of the potential mockingbird habitat on Gardner. In this plateau area, it is easy to catch mockingbirds and perform census walks. Opuntia cacti provide good mockingbird habitat and are especially abundant in the plateau area, but also north (ca 13 ha) and south (ca 12 ha) of the crater rim (Fig 3). Therefore, and because these are the areas where we observed mockingbirds most frequently, we believe that these three areas contain most mockingbird territories. Vegetation within the crater is scarce, as along some of the steep slopes, and only few mockingbirds have been observed to fly into or over the crater. Hence, the crater (Fig 3) is not considered suitable mockingbird habitat. To estimate population size for the entire island, we therefore believe that the number of birds estimated for the plateau area can roughly be tripled.

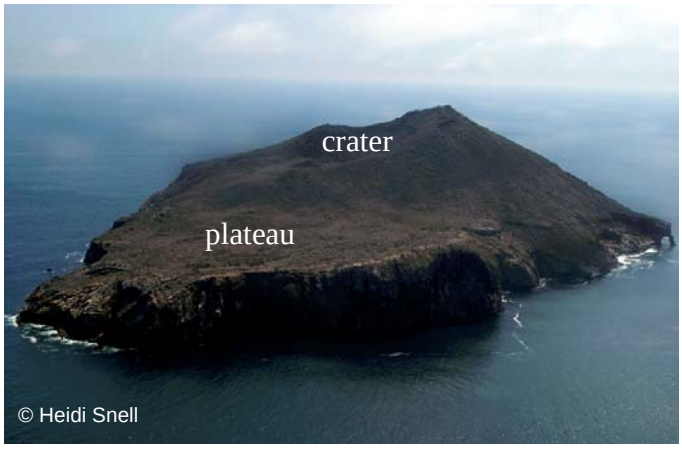

Fig 2: Aerial view of Gardner Islet.

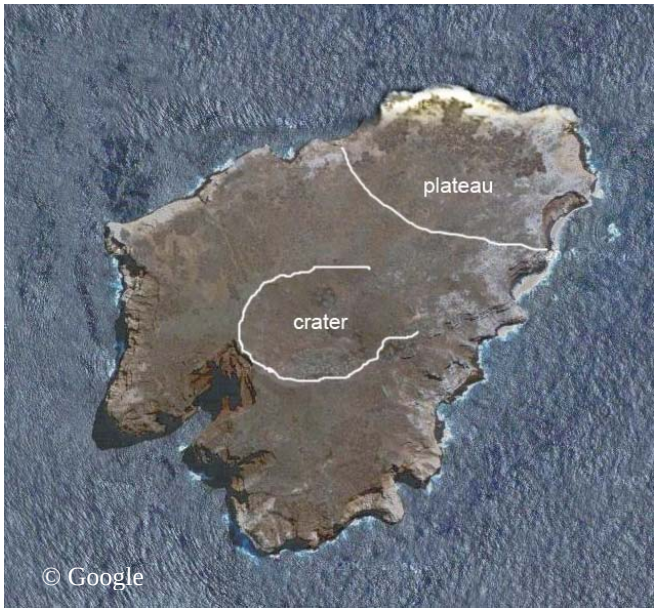

Fig 3: Satellite view of Gardner showing the plateau area where mockingbirds were banded and counted.

Census example on Gardner

For example, when we first visited Gardner to estimate population size in November 2006, we banded 23 individual mockingbirds in the plateau area in the days prior to our census. Assuming that no mortality occurred between capture and census, or that such effects can be neglected (a reasonable assumption for a time span of just a few days), M for this census equaled 23. We (Paquita Hoeck and Herbert Biebach) followed a track/transect from the west to the east of the plateau and back twice (see Fig 4), walking slowly but steadily and recording the location (GPS point) and time of every mockingbird we could spot. We used binoculars where necessary to see whether the bird was banded and, if so, recorded its color-band combination for individual identification (optional for the census, but necessary to estimate survival rates). During this census in November '06, we made 20 observations of color-banded individuals, 12 observations of unbanded individuals, and 2 observations for which we were unable to see if the birds had any bands at all. The census walk took us 2.5 hours and was approx. 2900 m long (Fig 4).

The 2 birds for which we couldn't tell whether they were banded or not, can be excluded from the census calculations because of the theoretically proportional chance of belonging to either group of birds (R or U).

Note that for the purpose of the census it does not matter whether the same individual was counted repeatedly, as long as the banded and unbanded birds are equally likely to be counted multiple times.

With these data at hand and the knowledge that 23 color-banded birds (M) were in the population, the total population-size in the plateau area can be estimated. We made 20 observations of banded birds (R) and 12 of unbanded birds (U), with M being 23. Hence, the estimation of the total population on the plateau is:

$N=MC/R=M(R+U)/R=23*(20+12)/20=36.8$

Assuming that the plateau area comprises about a third of the total mockingbird habitat on Gardner (Fig 3), we estimated a total population size of roughly 3*37=111 individuals on Gardner in Nov. '06 or 200-500 birds in 2008 and 2009 (Table 2).

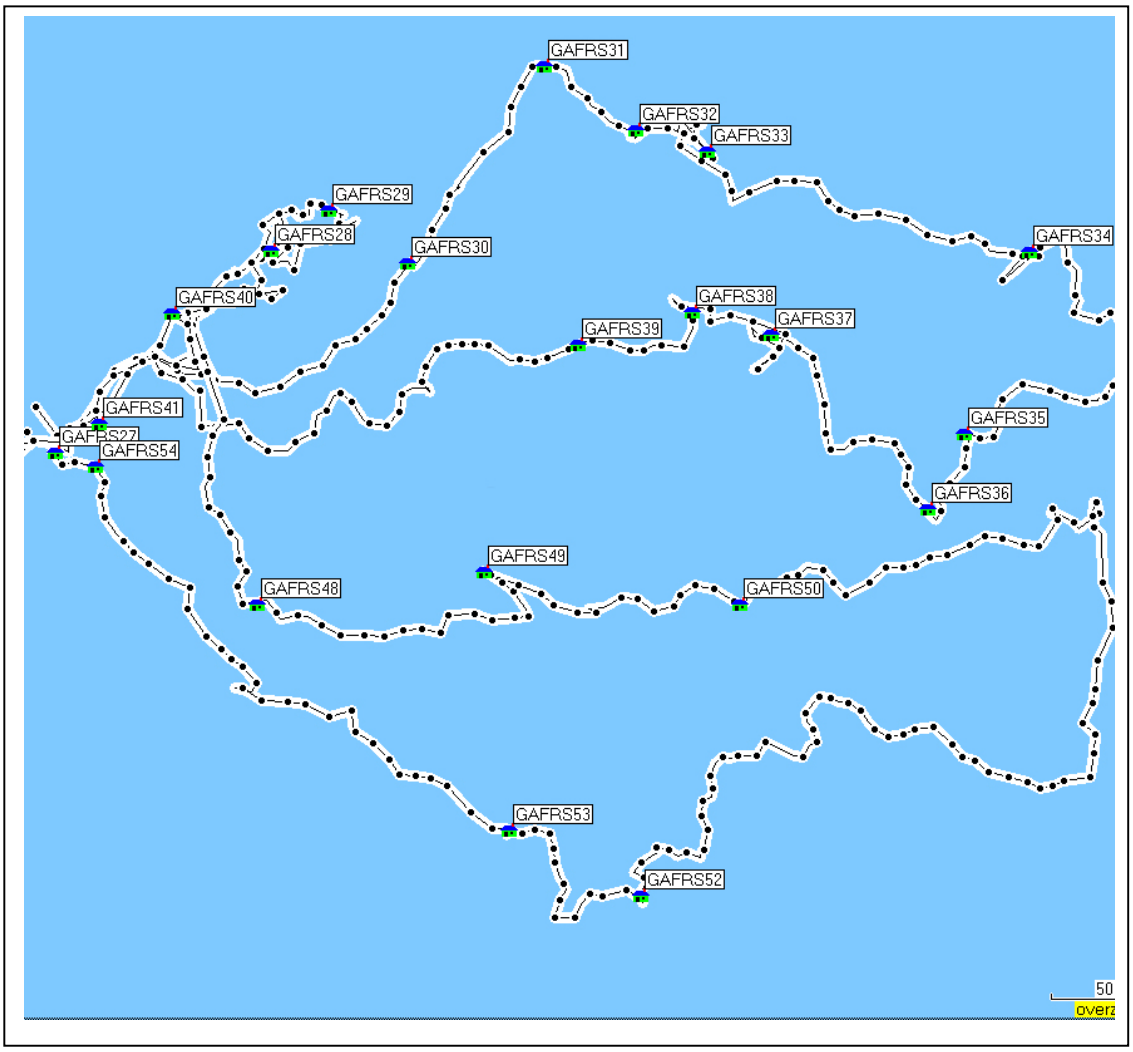

Fig 4: GPS track of the census performed on Gardner in Nov. 2006, covering most of the plateau area. Points at the left delineate the north-western coastline of the plateau, points at the top the northern end and points at the right the eastern edge of the plateau. The length of the track was 2900 meters and the census walk took about 2.5 hours.

Table 2: Population size estimates for Gardner-by-Floreana based on census results from 2006-2009.

Population size estimation for Gardner			
Individuals	**Nov. '06**	**Jan. '08**	**Feb. '09**
Banded (M)	23	35	40
Counted banded (R)	20	28	12
Counted unbanded (U)	12	29	36
Counted birds total (C)	32	57	48
Estimated population size, plateau area (N)	37	71	160
Estimated Nr of birds, total island (N*3)	111	213	480

Potential errors and limitations of the methodology

One fundamental assumption of this methodology is that the color-banded birds do not differ in their behavior from the unbanded birds. Because, obviously, only the banded birds were trapped prior to the census, we cannot exclude the possibility that these birds are either more or less likely to be observed during the census. If individuals previously caught for banding are particularly inquisitive and active birds, they might also be more likely to be seen again in the census. On the other hand, if trapping causes a change in behavior and results in the birds becoming less inquisitive or avoiding humans, they could be less likely to be counted. However, based on observational experience, we have no indication that previously trapped birds avoided us or changed their behavior towards us.

The number of birds recorded during a census may depend on many factors such as the visibility of the birds, observer qualification, number of observers, vegetation cover, weather, time of day, season or breeding phase of the birds. If these factors influence the banded birds in the same way as the unbanded birds, they should not affect the result of the population size estimation.

The reliability of the population estimate should increase with the proportion of banded birds relative to unbanded individuals. Equally important for the reliability of the population estimate is the relative number of observed banded versus unbanded birds during the census, which may depend on the length and intensity of the observations. Usually, there is an increasing variation in population size estimation with shortening of the census duration. Hence, speed and length of the census should be standardized. We walked the full length of the plateau (west-east) and shifted slightly in latitude (north-south) to assure broad coverage of the plateau region (Fig 4) and avoid walking the same track twice.

Also, to determine variance in population size estimation and reliability of the census, multiple census walks should be performed if possible, i.e. repeated censi on subsequent days (or in the morning and afternoon).

The assumption that the plateau area forms about one third of the suitable mockingbird habitat on Gardner should be tested in the future in order to improve the precision of the census results.

Estimating survival rates

Although individual identification of the banded birds is not necessary to obtain population size estimates, i.e. for the census, identifying the color-band combinations is crucial to estimate survival rates of the birds on Champion and Gardner. Therefore, if time and qualification of the personnel allow, individual color-band combinations should be recorded when ever possible.

REFERENCES

Buckland ST, Anderson DR, Burnham KP, Laake JL (1993) Distance Sampling: Estimating Abundance of Biological Populations. Chapman and Hall, London.

CDF (2008) *The Reintroduction of the Floreana Mockingbird to its Island of Origin* Charles Darwin Foundation & Galapagos National Park Service, Puerto Ayora, Galapagos.

CDF (2009) *Project Floreana 2009-2013: An integrated approach*, Puerto Ayora, Galapagos.

Grant PR, Curry RL, Grant BR (2000) A remnant population of the Floreana mockingbird on Champion island, Galapagos. *Biological Conservation* **92**, 285-290.

Harris MP (1973) Galapagos Avifauna. *Condor* **75**, 265-278.

Jiménez-Uzcátegui G (2006) Censo de cucuve de Floreana, Nesomimus trifasciatus 2006. Informe para la Estación Científica Charles Darwin y Servicio Parque Nacional Galápagos., Puerto Ayora, Galapagos, Ecuador.

Seber GAF (1982) *The Estimation of Animal Abundance and Related Parameters* Blackburn Press, Caldwel, New Jersey.

Vargas H (1996) Censo de Cucuves en el islote Champion. Informe técnico para la Estación Científica Charles Darwin., Puerto Ayora, Ecuador.

ADDITIONAL PUBLICATION

BACK TO THE FUTURE: MUSEUM SPECIMENS IN POPULATION GENETICS

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Back to the future: museum specimens in population genetics

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Museums and other natural history collections (NHC) worldwide house millions of specimens. With the advent of molecular genetic approaches these collections have become the source of many fascinating population studies in conservation genetics that contrast historical with present-day genetic diversity. Recent developments in molecular genetics and genomics and the associated statistical tools have opened up the further possibility of studying evolutionary change directly. As we discuss here, we believe that NHC specimens provide a largely underutilized resource for such investigations. However, because DNA extracted from NHC samples is degraded, analyses of such samples are technically demanding and many potential pitfalls exist. Thus, we propose a set of guidelines that outline the steps necessary to begin genetic investigations using specimens from NHC.

Introduction

Given that evolution is change over time, documenting and understanding temporal patterns has long been at the heart of evolutionary studies. In disciplines such as palaeontology, inferences about evolutionary processes are drawn from the analyses of temporal patterns in the fossil record. Similarly, our understanding of microevolutionary processes (i.e. changes in gene frequencies over time) has often involved the analyses of records taken over several years; Dobzhanksy's [1] early studies of microevolution among *Drosophila* used this approach, a tradition that continues among students of this model organism today [2]. However, such microevolutionary studies were often limited to certain taxa and questions because the time available to document temporal changes was often limited to a few generations.

How can these limitations be overcome? Long term studies, running over several decades, are one possibility and they are yielding fascinating insights, for example, into the role of reinforcement and character displacement in adaptive radiation and speciation [3,4]. Another approach, which gives longer time series, is to extend the data back in time using well preserved fossil samples or specimens from natural history collections (NHC). Here, we review the use of specimens from NHC for the study of evolutionary change. We aim to increase awareness of both the methodological limitations involved in using molecular methods with NHC specimens and their future potential.

We focus on studies of evolutionary change rather than the widespread use of NHC specimens in phylogenetics and phylogeography (e.g. Ref [5]) or pathogen origin and dynamics (e.g. Ref [6]). Similarly, we limit ourselves to studies of NHC specimens and do not consider studies of ancient DNA (Box 1; see [7–8] for excellent reviews on the latter). Although ancient DNA studies have yielded spectacular results [9,10], they will remain restricted to a relatively small set of species because the samples required for such work are rare and difficult to obtain. By contrast, NHC specimens generally cover a broader taxonomic range and are more easily obtained, thus enabling a wider range of questions and taxa to be studied.

NHC samples in conservation genetics

A large proportion of empirical studies of NHC samples published to date contrast past and recent genetic diversity in threatened and endangered populations or species (Table 1). Many now endangered or extinct populations and species became so within the past two centuries [11], a time period that coincides with the establishment of the majority of NHC (but see Ref [12]). As a result, specimens stored in NHC often represent the genetic diversity of populations shortly before significant anthropogenic influence. By inferring temporal changes in neutral genetic diversity, biologists can obtain estimates of the magnitude of anthropogenic influences on population sizes and connectivity (i.e. gene flow between populations) [13] and they can detect cryptic introductions or genetic introgression. Such insights could guide future conservation action.

Population declines and loss of genetic diversity

Low genetic variation in endangered populations is of conservation concern because genetic variation is the raw material required for future adaptive evolution. Low levels of genetic variation can be the consequence of recent population declines, or it can represent an ancestral state. Differentiating these two causes is therefore an important task in conservation biology and it can be achieved by comparing levels of genetic variability among NHC samples collected before a genetic bottleneck with those found in current populations.

One of the earliest studies to use such an approach was an investigation of an endangered population of the greater prairie chicken (*Tympanuchus cupido*) [14]. This study provided direct evidence for a human-induced decrease in genetic diversity over time: specific alleles present in the NHC samples were no longer present in the current population. Such 'ghost alleles' have subsequently

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Box 1. Ancient DNA versus DNA from NHC

NHC specimens have often been included in the definition of ancient DNA [7]. However, there are several differences between ancient DNA and that extracted from NHC. For example, ascertaining authenticity is crucial in ancient DNA work whereas in NHC-based studies authenticity is more easily ascertained by comparisons with results from high-quality DNA samples taken from extant populations. We therefore propose to make a distinction based on the criteria in Table I, even if there is not always a sharp contrast. Based on our criteria, working with DNA from NHC specimens is feasible when dedicated laboratory facilities are available for working with low-quality DNA and when the guidelines outlined in Boxes 3 and 4 can be fulfilled. By contrast, working with ancient DNA is methodologically considerably more demanding and, therefore, remains the domain of specialist groups and laboratories.

Table I. Differences between DNA extracted from NHC and ancient samples

	DNA from NHC	Ancient DNA
Sample origin	Museum, private and archive collections, herbarium	Archaeological and palaeontological sites, museum
Sample age	≤200 years	~100 000 years[a]
Sample size	Often large enough for population sample	Generally small[b]
Tissue types	Several (see Box 2)	Mainly hard tissue
Hominid samples	Seldom	Common
Main causes of DNA decay	Preservation methods, storage condition	Physical factors at sampling site, storage conditions
Level of DNA decay	High	Very high[c]
Variance of DNA quality among samples	Very high	High
Microsatellite amplification	Common	Seldom
Problems with authenticity	Moderate	Very high
Risk of contamination	High	Very high

[a]Sample age of one million years might be possible.
[b]The number of investigations with larger sample size is currently increasing.
[c]Although some ancient samples can yield higher DNA quality.

been reported in several bottlenecked animal populations. By contrast, other studies have reported stable genetic diversity despite declines in population size [24–26]. For example, no obvious loss of genetic diversity was detected among Canadian peregrine falcons (*Falco peregrinus*) despite a DDT induced bottleneck [25].

Inferring the genetically effective population size

The genetically effective population size (N_e) is a key parameter that determines the rate of loss of genetic variation owing to genetic drift. Measuring N_e is thus of interest in conservation biology, but is notoriously difficult [27]. However, several methods based on temporal samples have been developed, including forward probabilistic approaches and backwards coalescence approaches (reviewed in Ref [27]). Using microsatellites amplified from DNA extracted from historical samples, temporal estimates of N_e have been obtained in several different fish species [16,28–30], grizzly bears (*Ursus arctos*) [31], greater prairie chicken [14] and leopard frog (*Rana pipiens*) [32]. Some of these N_e estimates were lower than anticipated whereas others were higher. This pattern highlights the fact that including NHC samples in such analyses can prevent us from reaching misleading conclusions.

Changes in connectivity

Habitat fragmentation often leads to changes in population connectivity. With increasing population fragmentation, migration among populations and, thus, gene flow is expected to decrease. This leads to a reduction in genetic diversity within populations and an elevation of genetic differentiation among populations [18,33]. Such an increase in spatial genetic differentiation over a time period of six to seven generations has been reported for the endangered Spanish imperial eagle (*Aquila adalberti*) [33]. This finding suggests that future management policies should attempt to restore the ancestral panmictic situation through habitat restoration or translocations.

Detecting introductions and introgression

Specimens from NHC have been used also to detect new introductions and to assess the rate of genetic introgression into indigenous populations. For example, the cryptic invasion of a non-native genotype of the common reed (*Phragmites australis*) in North America during the past century was detected by sequencing samples collected worldwide at two noncoding regions of the chloroplast [34]. Similarly, archival fish scales have been used to quantify the genetic consequences for wild fish populations of stocking or of aquaculture escapees [35,36]. Stocking of Spanish Atlantic salmon (*Salmo salar*) since the 1970s appears to have increased the mitochondrial DNA diversity of four endangered populations. However, it failed to halt the ongoing population decline [35].

NHC samples in evolutionary biology

Fisher and Ford [37] provided an early example of the use of NHC specimens to study evolutionary change directly. Their 1947 study of the spread of the *medionigra* gene among the scarlet tiger moth (*Callimorpha dominula*) provided clear evidence for gene frequency change owing to natural selection. Since then, surprisingly few studies have taken advantage of the evolutionary history preserved in NHC samples to investigate the molecular footprint of selection. One such study investigated the evolutionary genetic mechanisms that underlie the rapid evolution of insecticide resistance in the blowfly (*Lucilia cuprina*) in Australia [38]. Sequence data from 16 pinned NHC specimens collected before the first use of organophosphate insecticides revealed that the rapid evolution of insecticide resistance was because of the pre-existence of mutant alleles in the historical gene pool and that the

Table 1. A selection of studies contrasting past and recent genetic diversity using specimens from NHC[a,b]

Species	Marker[c]	Time span[d]	Sample[e]	N[f]	Summary	Refs
Plants						
Common reed *Phragmites australis*	1400 bp cpDNA	Before 1910; after 1960	5/2	62/283	Cryptic invasion of non-native genotype	[34]
Marsh orchid *Anacamptis palustris*	250–280 bp plastid DNA	1832–recent	5/4	89/316	Genetic variation in time and space	[72]
Insects						
Sheep blowfly *Lucilia cuprina/sericata*	287 bp *LcαE7*	1930–1990	-/2	16/35	Evolution of insecticide resistance	[38]
Adonis blue *Polyommatus bellargus*	4	1897–1999	1/2	20/74	High genetic drift in an insect population	[73]
Fish						
Northern pike *Esox lucius*	7	1961–1993	1/3	196/72	Temporal N_e estimation of introduced population	[29]
Atlantic salmon *Salmo salar*	6	1913–1994	7/4	228/90	Genetic diversity between and within extant and extinct populations	[74]
Newfoundland cod *Gadus morhua*	6	1964–1994	5/3	574/570	Stability of genetic structure despite population size decline	[24]
Brown trout *Salmo trutta*	8	1910–1992	5/3	191/311	Estimation of N_e and temporal stability of genetic structure	[28]
	9	1945–2000	2/7	146/397	Genetic effects of stocking domestic trout into wild populations	[36]
New Zealand snapper *Pagrus auratus*	7	1950–1998	2/5	372/96	Genetic diversity and low N_e in two overexploited populations	[16]
Steelhead trout *Oncorhynchus mykiss*	7	1958–1998	3/4	180/90	Temporal diversity and N_e for three populations	[30]
Brown trout *S. trutta*	6; 1 *Satr-UBA*[g]	1958–1995	1/5	232/50	Temporal variation of MHC class I gene during aquaculture activities	[68]
Atlantic salmon *S. salar*	1409 bp RFLP	1948–2002	4/5	592/125	Effects of stocking on mitochondrial diversity	[35]
Amphibians						
Leopard frog *Rana pipiens*	7	1971–2001	5/2	204/188	N_e and temporal stability of genetic structure	[32]
Birds						
Greater prairie chicken *Tympanuchus cupido*	6	1930s–recent	4/2	15/127	Genetic diversity of pre- and post-bottleneck populations	[14]
Mauritius kestrel *Falco punctatus*	12	1830–recent	7/2	52/250	Genetic monitoring of a pesticide-induced bottleneck	[17]
Greater prairie chicken *Tympanuchus cupido*	384 bp; 6	1951-2000	4/2	125/81	N_e and temporal genetic variation in bottlenecked populations	[18]
White-headed duck *Oxyura leucocephala*	192 bp	1861–2003	-/2	67/46	Loss of genetic diversity	[21]
Peregrine falcon *Falco peregrinus*	405 bp; 11	1885–2004	-/2	95/184	Pesticide-induced bottleneck in Canadian falcons	[25]
Spanish imperial eagle *Aquila adalberti*	345 bp; 10	1860-recent	1/2	34/79	Effects of fragmentation on spatiotemporal genetic structure	[33]
Mammals						
Kangaroo rat *Dipodomys panamintinus*	225 bp	1911–1988	3/2	49/63	Continuity of spatial and temporal mtDNA diversity	[75]
Northern hairy-nosed wombat *Lasiorhinus krefftii*	90 bp; 8	1883–1994	3/2	5/29	Genetic variation of extinct and extant populations	[76]
Hector's dolphin *Cephalorhynchus hectori*	206 bp	1870–1998	2/4	55/108	Loss of genetic diversity owing to fishery-related mortality	[20]
European otter *Lutra lutra*	9	1883–1993	3/3	67/58	Genetic consequences of population decline	[26]
Elephant seal *Mirounga angustirostris*	116 bp; 4	1500s–1990s	1/2	22/185	Effects of bottleneck on genetic diversity and on symmetry of bilateral characters	[19]
Grizzly bear *Ursus arctos*	8	1912–1999	1/3	110/136	N_e of Yellowstone grizzly	[31]
Common hamster *Cricetus cricetus*	240 bp; *DRB*[h]	1924–2000	2/3	20/31	Loss of MHC diversity in the *DRB* exon 2	[67]
Grey wolf *Canis lupus*	229 bp; 15+4[i]	1829–recent	4/2	33/22	Genetic variability and migration during population decline	[77]
	425 bp	1856–recent	-/2	32/399	Genetic variability and population size of extirpated US wolf populations	[23]
Arctic fox *Alopex lagopus*	292 bp; 5	1831–2004	2/2	21/41	Demographic bottleneck	[15]
Red fox *Vulpes vulpes*	354 bp	1911–2002	5/2	29/35	Genetic evidence for the persistence of Sierra Nevada red fox	[22]

[a]Within taxonomic groups, the order is by year of publication.
[b]Abbreviations: cpDNA, chloroplast DNA; MHC, major histocompatibility complex; RFLP, restricted fragment length polymorphisms.
[c]Unless otherwise noted, sequence length in base pairs (bp) stands for mitochondrial DNA and the single digit for the total number of microsatellites loci applied.
[d]The oldest and the most recent year of sampling.
[e]Number of populations and (/) number of temporal samples.
[f]Sample size of historical and (/) most recent samples.
[g]A microsatellite locus embedded in the MHC class I locus of brown trout.
[h]Gene that presents extracellular proteins to T lymphocytes.
[i]Refers to Y chromosome microsatellites.

Box 2. Tissue samples from natural history collections for genetic studies

Several types of tissue have been applied to obtain genetic data from NHC specimens. Here, we provide an overview of the most common tissue types used and their advantages and disadvantages in terms of potential damage to the specimens and the expected DNA quality.

Hides and skins
Epithelium tissue is a preferred source of DNA because damage is often insignificant and sampling is straightforward. Different preservation methods can, however, create substantial DNA degradation and PCR inhibition [48], which causes a high variance in DNA quality among samples. Superior DNA can be extracted from claws on hides of mammalian specimens [49] or from toe pads of birds [78].

Bones
DNA in bones is generally better protected than in epithelium tissue. However, DNA extraction is laborious because the material needs to be ground with a drill or a mortar. The use of maxilloturbinal bone material (i.e. thin bones inside the nasal cavities) might minimize damage to specimens [45], except where these bones are important for morphological analyses.

Teeth
DNA from teeth is generally well preserved given the hard tissue protecting it. Extraction protocols for teeth [51,77] are often identical to bones, although the efficiency can vary among different protocols [79]. To prevent the destruction of the whole tooth, only the root tip can be removed or the material following drilling inside the root cavity can be recovered. Nondestructive extraction methods have been developed [44,46].

Feathers
DNA has been extracted from the base of the feather calamus or the blood clot from the superior umbilicus. The latter is thought to yield higher amounts of DNA [80]. Careful selection of feathers results in little damage to the appearance of the specimen and removed feathers can be reattached [81].

Fish scales
Initially collected for use in age determination of individuals, several extraction protocols have been developed for scales [35,40]. Because they are plucked from fresh material, dermic and epidermic cells remain attached to the scale and dry up sufficiently fast to prevent DNA degradation [74].

Pinned insects
DNA can be extracted from pinned specimens using the whole insect or body parts [73]. Protocols conferring no external damage have recently been developed [82]. Different killing methods and storage conditions can affect the recovered DNA [83].

Fluid specimens
Many NHC specimens are stored in aqueous formalin. DNA extraction and PCR amplification from formalin-fixed specimens, however, is particularly difficult [50,54]. Formalin storage can cause frequent nucleotide misincorporations [50,55]. Particular care is needed to detect potential cross-contamination among samples because several specimens are often stored within the same fluid or because fluid among different storage containers could have been exchanged.

Herbarium specimens
Leaf tissue [34,84] and seeds [85] from plants dried and stored under controlled conditions and not preserved with chemicals can provide useful amounts of nuclear and chloroplast DNA [84].

Ethnographic artefacts
Animal and plant parts are also preserved in artefacts of ethnographic collections. Such collections can contain specimens that were collected before the major collection activities of NHC and thus might yield even longer time series (J. Groombridge, personal communication).

associated selective sweep has led to a significant loss of genetic variability [38]. This study illustrates the power of harnessing modern molecular genetic approaches with long time series data, either through the use of NHC samples or through long term studies [2].

Pitfalls and precautions

NHC hold an unchallenged wealth of specimens that reflect past and current biodiversity of our planet. However, molecular studies based on historical samples are challenging because genotype and sequence data obtained by PCR are often error prone. Consequently, precautions are needed to guarantee reliable genetic data.

Incomplete specimen records and small sample size

Unsurprisingly, most specimens of NHC collections were not assembled with the aim of carrying out genetic studies. As described by Fisher and Ford in their scarlet tiger moth study [37], this can considerably limit the utility of NHC collections for genetic studies. Collectors were likely to be biased in their sampling effort, particularly in relation to colour morphs and other phenotypic varieties. This might be less problematic when neutral genetic markers rather than functional genes are studied, but sampling localities, age and sex of the animals can still be biased. In addition, records for specimens from NHC are frequently incomplete, imprecise, missing or incorrect [39].

In a few instances, archival collections are reasonably comprehensive. For example, commercially and recreationally important fish species are often represented in systematic and continuous collections of scales or otolithes, sampled since the beginning of the 19th century to determine the age of individuals [40]. Few other taxa are represented by such comprehensive collections. Given that the number of historical specimens is limited, sample sizes can only be increased by searching longer for appropriate material, which is likely to exceed the time and effort spent on sampling modern material.

A related problem is that some specimens might not be suitable for molecular studies, because DNA needs to be physically extracted from tissue, which causes potential damage to the specimen (Box 2). Fifteen years ago, this problem led to a debate over the benefits and misuse of molecular studies based on NHC [41,42] and prompted several museums to establish guidelines for the use of specimens from their collections (Box 3). However, given the small amount of tissue now needed for extracting sufficient amounts of DNA for PCR amplification and the development of less or nondestructive DNA extraction methods [43–46], we believe that few NHC specimens should be out of bounds for molecular studies.

Molecular work with low-quality DNA

Numerous biological, physical and chemical factors affect the DNA quality of specimens from NHC. Most of these factors have been extensively described for ancient DNA work (reviewed in Refs [7,8]) and are also relevant for NHC, such as endogenous nuclease activity and hydrolytic damage. As a consequence, DNA extracted from historical material can be expected to be highly degraded and hence highly diluted similar to DNA derived from noninvasive

Box 3. Guidelines for genetic studies using natural history collections: study design

These guidelines are intended to outline the steps necessary to begin genetic investigations using specimens from NHC. They are divided into two sections covering the general (this Box) and the laboratory aspects of such projects (Box 4).

Collaboration with natural history museums
Unless specified otherwise by museums, researchers should provide a short project proposal that includes justification for the required material and evidence of the experience with such work. An agreement between the researchers and the museum is advantageous to clarify storage of and access to surplus extracted DNA and the future use of the genetic data. Museums should be regularly informed, acknowledged in publications and supplied with reprints of publications at the end of the study.

Sample selection
Records of specimens need to be verified to account for potential identification errors (e.g. imprecision or error in the location of a record [39]). Depending on the geographical and temporal scale of the intended study, specimens with imprecise records should be excluded. Only small tissue samples should be taken, causing the least amount of damage while providing a good likelihood of extracting sufficiently preserved DNA (Box 2).

Pilot study
A pilot study is invaluable to evaluate PCR amplification success and, where possible, to quantify the copy number of the target DNA. In addition, the frequency of genotyping errors in microsatellites and single base pair errors in sequence data should be assessed (Box 4). These results will clarify whether the scientific goals are realistic given the number of samples available, the sample quality and the molecular methods applied and will help to justify damage to additional specimens.

Sceptical attitude to own results
As records of specimens might be incorrect, results from genetic analyses should be evaluated carefully. Any samples for which specimen records and genetic data do not match or do not make biological sense should be treated with particular care. Moreover, results that might indicate methodological artefacts rather than biological findings have to be interpreted carefully.

Box 4. Guidelines for genetic studies using natural history collections: laboratory work

The main laboratory criteria relevant for working with specimens from NHC are based, in part, on those originally proposed for ancient DNA work [7,8]. However, there are some significant differences between ancient DNA and DNA from NHC (Box 1), which lead to the following guidelines.

Choosing appropriate genetic markers
Because DNA extracted from NHC specimens is highly degraded, amplicons of sequence data and microsatellite loci ideally should be <200 bp in size. All primers should be specifically designed for the species of interest to yield the best possible PCR efficiency and should have been extensively tested in modern samples. Species specific primers often can be redesigned within the flanking region of microsatellite loci and PCR multiplexing can be advantageous to delay the depletion of template DNA. The amplification of overlapping variable sequences of mitochondrial DNA will further diminish the chance that the sequence derives from a nuclear insertion.

Isolation of laboratory work area
Cross-contamination and contamination with exogenous DNA is a key concern when working with historical DNA. Therefore, DNA extraction and PCR preparation should be carried out in a dedicated and isolated laboratory with one way movement of DNA out of the laboratory. Only reagents exclusively purchased and maintained for working within such an environment must be used and extensive decontamination (e.g. UV radiation) of the laboratory, working surfaces and equipment is essential. Because the variation in the quantity of recovered DNA among NHC specimens can be very large, the number of simultaneously extracted samples needs to be kept small. The interspersion of samples that are likely to yield different haplotypes can further improve the detection of cross-contamination.

Negative control for extraction and PCR
Negative extraction and PCR controls need to be included to detect potential contamination in reagents and cross-contamination between samples.

Appropriate molecular behaviour
PCR amplification intensity of historical DNA is inversely related to product size, and products >500 bp are unusual. Deviations from such appropriate behaviour should be cause for careful checking and repetitions.

Reproducibility of genetic data
Several biological and nonbiological processes can damage DNA causing single nucleotide misincorporations [52,53]. Independent PCR and sequencing of unknown haplotypes are therefore required and particularly important sequences should be verified by cloning. Genotyping errors in microsatellites such as allelic dropout and false alleles are widespread in historical samples [15,51] and can be more common among loci with longer fragment size and among older samples [51]. Several independent PCR replications are required to attain a sufficiently high genotyping reliability [47]. Alternatively, the template DNA concentration can be estimated by quantitative PCR to adjust the number of PCR replications [56].

sampling [47]. In addition, different preservation methods can negatively affect the ability to extract, amplify and sequence DNA [48–50]. PCR amplification of historical DNA is, therefore, generally restricted to short amplicons (<200 bp) and is further vulnerable to contamination by recent DNA and PCR products from the study species. Because preservation methods can vary considerably, the variance expected in DNA quality among samples of similar age can be large and the risk of cross contamination is considerable. Consequently, sample age is not the only important factor that affects DNA quality ([44], but see Ref [51]). Molecular work with DNA from NHC specimens requires special precautions, including an isolated and dedicated laboratory environment (Box 4).

The cumulative damage to the DNA can also cause incorrect bases to be inserted during enzymatic amplification. The main source for these alterations are single nucleotide misincorporations ([7,52], and references therein). C to T transitions are the main type of such alterations that occur when an erroneous DNA strand is replicated during the first cycle of a PCR. Initially thought to be limited to ancient DNA, nucleotide misincorporations have recently been reported in studies based on specimens from NHC [15,52,53]. Estimates so far range from 17% to 21% of sequences that show one or more errors [15,53]. Special cases are formalin-preserved specimens, in which sequence alterations can occur at even higher frequencies [50,54,55]. Misincorporations might look like new alleles or new sequences and can therefore lead to systematic overestimation of the genetic diversity of past populations [53]. Consequently, repeated sequencing of independent PCR

products and cloning of important sequences are needed to ensure reliable genetic data [52].

The highly degraded and, therefore, diluted nature of DNA extracted from NHC specimens can cause a significant rate of genotyping errors when biparental genetic markers, such as microsatellites, are amplified by PCR. Two types of genotyping errors are likely: allelic dropout and false alleles. Allelic dropout is the stochastic nonamplification of one of the two alleles present at a heterozygote locus [47]. False alleles are PCR amplification artefacts that occur by the slippage of the taq polymerase during the first cycles of the PCR [47]. False alleles are less frequent than allelic dropouts. Because the rate of both types of error is inversely correlated with the concentration of the extracted DNA [47,51,56], a high frequency of allelic dropout can be expected in samples from NHC with low DNA quality. Consequently the genetic diversity measured will be systematically underestimated in historical samples compared with modern samples. This contrasts with the overestimation in sequence data that can occur owing to misincorporations and owing to false alleles. Several approaches have been proposed to achieve higher genotyping reliability for microsatellites in samples with low DNA quantity, including a predefined number of repeated and independent PCR amplifications [47] and the initial quantification of the template DNA concentration [51,56] by quantitative PCR assays.

Appropriate standards for reliable genetic data

On the one hand, the findings of molecular studies based on NHC can be misleading when ignoring the effects of sequence alterations and genotyping errors. On the other hand, it might be difficult to fulfil stringent standards, for example, when only a minute amount of DNA from a precious specimen is available and, as a consequence, only a small number of PCR reactions can be performed. Thus, although we propose guidelines for molecular genetic studies based on NHC samples (Boxes 3 and 4), we caution against sweeping, global standards. Instead, and similar to the approach advocated by Gilbert *et al.* [57] for assessing the authenticity of ancient DNA, we recommend a flexible approach, cognitive of the problems particular to every study.

Investigations using historical material need to be carefully assessed on a case-by-case basis by scrutinizing the magnitude of potential errors in historical genetic data in relation to the general findings of the investigation. For example, a minor and overlooked rate of allelic dropout might have a small effect on estimates of allele frequencies but could significantly alter the outcome of relatedness analyses with NHC samples. In particular, a sceptical attitude [7] towards ones own results is needed when findings can equally well be explained by methodological artefacts and biological processes.

Prospects

In the near future, advances in molecular technologies will enable access to more and more genetic information from specimens archived in NHC. This progress will allow us to shift from neutral genetic markers to specific genes under selection.

Gaining more genetic information from NHC samples

Mitochondrial sequences and microsatellites have been the preferred genetic markers in studies using NHC, which reflects the present genetic tools of choice for non-model organisms in ecology and evolution. Recently, single nucleotide polymorphisms (SNPs) have been put forward as an alternative to microsatellites [58]. SNPs have several advantages, including a known mutation model and a higher genotyping efficiency. Furthermore, they are suitable for highly degraded DNA because genotyping requires only short target DNA sequences (<100 bp) and protocols for genotyping of SNPs have been developed for degraded DNA [59]. However, the discovery of SNPs, that is, the selection of representative samples used for finding polymorphic sites, is crucial and can significantly bias the estimates of genetic variation within and between populations ([58], and references therein). To avoid this ascertainment bias, samples from different time periods and therefore also from historical samples could be used for the discovery of SNPs. Obviously, the degraded nature of historical DNA and the potential of nucleotide misincorporations will make this difficult. At present, the trade-off between these methodological constraints is unresolved and new molecular methods are needed to deploy the full potential of SNPs in NHC based studies. One such method is parallel pyrosequencing with a 454 instrument [60], which has been used in ancient DNA studies [61]. Overall, we believe that SNPs could soon become the marker of choice in studies based on NHC.

Genotyping and sequencing can rapidly deplete the precious and often limited amounts of DNA extracted from NHC specimens. This process can partly be delayed by multiplexing several loci within one PCR reaction, an approach now regularly used in microsatellite and SNP genotyping [59]. PCR multiplex amplification has also been used to obtain the complete sequence of the mitochondrial genome from the woolly mammoth (*Mammuthus primigenius*) from a 200 mg bone sample [62]. However, a complete and representative amplification of the limited DNA is preferred, which would provide a nearly unlimited copy number of genomic DNA for future molecular work. This could be achieved by applying whole-genome amplification (WGA) techniques. Several different WGA protocols have been developed to amplify the genomic DNA from minute amounts of template DNA, that is, from a few cells [63]. Most of these protocols rely on high quality template DNA, although protocols tolerant of degraded DNA have recently been developed [64]. We hope that in the near future novel WGA protocols will be adopted for use with NHC specimens.

Conservation genetics

DNA extracted from NHC specimens adds an important temporal dimension to the genetic study of endangered species and will remain pivotal in conservation genetics to assess phylogenetic positions and evolutionary significant (or management) units and to infer changes in population size and structure. Furthermore, contrasting past and recent genetic variability could serve as a retrospective approach of genetically monitoring populations for conservation and management [65]. Currently, there is growing

interest in understanding gene flow in a heterogeneous landscape by combining landscape and ecological data with individual based genetic data [66]. Historical samples might prove particularly valuable in this context because they enable direct tests of hypotheses concerning human-induced changes in gene flow patterns over time.

Beyond neutral markers

The main body of population genetic research using samples from NHC to date has focussed on temporal changes in genetic diversity of neutral genetic markers. By contrast, only a few NHC-based studies have identified allele frequencies of coding DNA sequences [38,67] or of microsatellites closely linked to sequences potentially under selection [68]. The relative ease with which one can now assay candidate genes and an increasingly large number of genetic markers makes it possible to study (natural) selection directly using temporal samples. Comparing the genetic variation from different time periods can be a powerful tool to detect molecular signatures of selection or environmental causes of selection. This has been exemplified by documenting insecticide resistance [38], evolutionary response to climate change [2] and human-induced allelic selection in maize [69].

Nevertheless, these approaches are not without caveats. Temporal variation of allele frequencies of a gene suspected to be under selection (or of a marker linked to it) occur because of selection, genetic drift or a combination of both. Consequently, statistical methods that separate the effects of selection from drift are required and these are often sensitive to assumptions about the demography of the populations [70]. Furthermore, different selection regimes can yield the same allele frequency dynamics [71], thus limiting the details that can be inferred from allele frequency dynamics data alone. Statistical analyses of selection are, however, a very active research field [70] and future methodological developments are likely to open up more possibilities.

Conclusions

Analyses of DNA from NHC samples have played an important role in conservation genetics by identifying processes that have shaped current levels of genetic diversity. A strong taxonomic bias is apparent among the studies to date (Table 1). Vertebrates and particularly fish predominate, whereas studies on plants and invertebrates are surprisingly rare. This bias might, in part, have arisen from biases in sample availability, conservation interests and methodological constraints, but the lack of more plant studies remains puzzling.

As NHC-based genetic work is coming of age, studies might fruitfully shift from investigating neutral genetic variation to studying the interplay of selection and drift. Although pioneered by Fisher and Ford 60 years ago [37], such direct studies of evolutionary change through the use of NHC samples are only beginning. Although technically demanding, such studies will be worth all the effort. Fisher and Ford [37] put it nicely: 'The spread of a gene in natural conditions is an event which repays detailed study since it provides an opportunity for examining evolution in progress. It has long been apparent to us that a careful watch should be kept for this occurrence and that whenever found it should, if possible, be analysed from two distinct points of view, ecological and genetic – a technique which has so far received much less attention than it deserves.' Combined with recent samples and, where possible, with ecological data that represent both current and historical time periods [39], NHC specimens provide a largely untapped resource for such investigations.

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References

1 Dobzhansky, T. (1943) Genetics of natural populations IX. Temporal changes in the composition of populations of *Drosophila pseudoobscura*. *Genetics* 28, 162–186
2 Balanya, J. *et al.* (2006) Global genetic change tracks global climate warming in *Drosophila subobscura*. *Science* 313, 1773–1775
3 Silvertown, J. *et al.* (2006) The Park Grass Experiment 1856-2006: Its contribution to ecology. *J. Ecol.* 94, 801–814
4 Grant, P.R. and Grant, B.R. (2006) Evolution of character displacement in Darwin's finches. *Science* 313, 224–226
5 Shapiro, B. *et al.* (2002) Flight of the dodo. *Science* 295, 1683
6 Bearchell, S.J. *et al.* (2005) Wheat archive links long-term fungal pathogen population dynamics to air pollution. *Proc. Natl. Acad. Sci. U. S. A.* 102, 5438–5442
7 Paabo, S. *et al.* (2004) Genetic analyses from ancient DNA. *Annu. Rev. Genet.* 38, 645–679
8 Hofreiter, M. *et al.* (2001) Ancient DNA. *Nat. Rev. Genet.* 2, 353–359
9 Hadly, E.A. *et al.* (2004) Genetic response to climatic change: insights from ancient DNA and phylochronology. *PLoS Biol.* 2, e290 (http://biology.plosjournals.org)
10 Shepherd, L.D. *et al.* (2005) Microevolution and mega-icebergs in the Antarctic. *Proc. Natl. Acad. Sci. U. S. A.* 102, 16717–16722
11 Ceballos, G. and Ehrlich, P.R. (2002) Mammal population losses and the extinction crisis. *Science* 296, 904–907
12 Paxinos, E.E. *et al.* (2002) Prehistoric decline of genetic diversity in the nene. *Science* 296, 1827
13 Roy, M.S. *et al.* (1994) The use of museum specimens to reconstruct the genetic-variability and relationships of extinct populations. *Experientia* 50, 551–557
14 Bouzat, J.L. *et al.* (1998) The ghost of genetic diversity past: historical DNA analysis of the greater prairie chicken. *Am. Nat.* 152, 1–6
15 Nyström, V. *et al.* (2006) Genetic consequences of a demographic bottleneck in the Scandinavian arctic fox. *Oikos* 114, 84–94
16 Hauser, L. *et al.* (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). *Proc. Natl. Acad. Sci. U. S. A.* 99, 11742–11747
17 Groombridge, J.J. *et al.* (2000) 'Ghost' alleles of the Mauritius kestrel. *Nature* 403, 616
18 Johnson, J.A. *et al.* (2004) Temporal changes in allele frequencies and low effective population size in greater prairie-chickens. *Mol. Ecol.* 13, 2617–2630
19 Hoelzel, A.R. *et al.* (2002) Impact of a population bottleneck on symmetry and genetic diversity in the northern elephant seal. *J. Evol. Biol.* 15, 567–575
20 Pichler, F.B. and Baker, C.S. (2000) Loss of genetic diversity in the endemic Hector's dolphin due to fisheries-related mortality. *Proc. R. Soc. Lond. B Biol. Sci.* 267, 97–102
21 Munoz-Fuentes, V. *et al.* (2005) Population structure and loss of genetic diversity in the endangered white-headed duck, *Oxyura leucocephala*. *Conserv. Genet.* 6, 999–1015
22 Perrine, J.D. *et al.* (2007) Genetic evidence for the persistence of the critically endangered Sierra Nevada red fox in California. *Conserv. Genet.* 8, 1083–1095
23 Leonard, J.A. *et al.* (2005) Legacy lost: genetic variability and population size of extirpated US grey wolves (*Canis lupus*). *Mol. Ecol.* 14, 9–17

24 Ruzzante, D.E. *et al.* (2001) Stability in the historical pattern of genetic structure of Newfoundland cod (Gadus morhua) despite the catastrophic decline in population size from 1964 to 1994. *Conserv. Genet.* 2, 257–269
25 Brown, J.W. *et al.* (2007) Appraisal of the consequences of the DDT-induced bottleneck on the level and geographic distribution of neutral genetic variation in Canadian peregrine falcons, *Falco peregrinus*. *Mol. Ecol.* 16, 327–343
26 Pertoldi, C. *et al.* (2001) Genetic consequences of population decline in the European otter (Lutra lutra): an assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. *Proc. R. Soc. Lond. B Biol. Sci.* 268, 1775–1781
27 Wang, J.L. (2005) Estimation of effective population sizes from data on genetic markers. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 1395–1409
28 Hansen, M.M. *et al.* (2002) Long-term effective population sizes, temporal stability of genetic composition and potential for local adaptation in anadromous brown trout (*Salmo trutta*) populations. *Mol. Ecol.* 11, 2523–2535
29 Miller, L.M. and Kapuscinski, A.R. (1997) Historical analysis of genetic variation reveals low effective population size in a northern pike (*Esox lucius*) population. *Genetics* 147, 1249–1258
30 Heath, D.D. *et al.* (2002) Temporal change in genetic structure and effective population size in steelhead trout (*Oncorhynchus mykiss*). *Mol. Ecol.* 11, 197–214
31 Miller, C.R. and Waits, L.P. (2003) The history of effective population size and genetic diversity in the Yellowstone grizzly (*Ursus arctos*): Implications for conservation. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4334–4339
32 Hoffman, E.A. *et al.* (2004) Effective population sizes and temporal stability of genetic structure in Rana pipiens, the northern leopard frog. *Evolution Int. J. Org. Evolution* 58, 2536–2545
33 Martinez-Cruz, B. *et al.* (2007) Population fragmentation leads to spatial and temporal genetic structure in the endangered Spanish imperial eagle. *Mol. Ecol.* 16, 477–486
34 Saltonstall, K. (2002) Cryptic invasion by a non-native genotype of the common reed, *Phragmites australis*, into North America. *Proc. Natl. Acad. Sci. U. S. A.* 99, 2445–2449
35 Ciborowski, K.L. *et al.* (2007) Stocking may increase mitochondrial DNA diversity but fails to halt the decline of endangered Atlantic salmon populations. *Conserv. Genet.* DOI: 10.1007/s10592-007-9286-2 (http://www.springerlink.com/content)
36 Hansen, M.M. (2002) Estimating the long-term effects of stocking domesticated trout into wild brown trout (*Salmo trutta*) populations: an approach using microsatellite DNA analysis of historical and contemporary samples. *Mol. Ecol.* 11, 1003–1015
37 Fisher, R.A. and Ford, E.B. (1947) The spread of a gene in natural conditions in a colony of the moth *Panaxia dominula L. Heredity* 1, 143–174
38 Hartley, C.J. *et al.* (2006) Amplification of DNA from preserved specimens shows blowflies were preadapted for the rapid evolution of insecticide resistance. *Proc. Natl. Acad. Sci. U. S. A.* 103, 8757–8762
39 Graham, C.H. *et al.* (2004) New developments in museum-based informatics and applications in biodiversity analysis. *Trends Ecol. Evol.* 19, 497–503
40 Nielsen, E.E. *et al.* (1997) Analysis of microsatellite DNA from old scale samples of Atlantic salmon *Salmo salar*: A comparison of genetic composition over 60 years. *Mol. Ecol.* 6, 487–492
41 Graves, G.R. and Braun, M.J. (1992) Museums - storehouses of DNA. *Science* 255, 1335–1336
42 Schram, F.R. (1992) Museum collections - why are they there? *Science* 256, 1502
43 Hutchinson, W.F. *et al.* (1999) A nondestructive technique for the recovery of DNA from dried fish otoliths for subsequent molecular genetic analysis. *Mol. Ecol.* 8, 893–894
44 Rohland, N. *et al.* (2004) Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. *Biotechniques* 36, 814–821
45 Wisely, S.M. *et al.* (2004) A technique for sampling ancient DNA that minimizes damage to museum specimens. *Conserv. Genet.* 5, 105–107
46 Pichler, F.B. *et al.* (2001) Nondestructive DNA extraction from sperm whale teeth and scrimshaw. *Mol. Ecol. Notes* 1, 106–109
47 Taberlet, P. *et al.* (1999) Noninvasive genetic sampling: look before you leap. *Trends Ecol. Evol.* 14, 323–327
48 Hall, L.M. *et al.* (1997) Association of enzyme inhibition with methods of museum skin preparation. *Biotechniques* 22, 928–934
49 Hedmark, E. and Ellegren, H. (2005) Microsatellite genotyping of DNA isolated from claws left on tanned carnivore hides. *Int. J. Legal Med.* 119, 370–373
50 Tang, E.P.Y. (2006) *Path to Effective Recovering of DNA from Formalin-Fixed Biological Samples in Natural History Collections Workshop Summary,* The National Academies Press
51 Wandeler, P. *et al.* (2003) Patterns of nuclear DNA degeneration over time - a case study in historic teeth samples. *Mol. Ecol.* 12, 1087–1093
52 Stiller, M. *et al.* (2006) Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. *Proc. Natl. Acad. Sci. U. S. A.* 103, 13578–13584
53 Sefc, K.M. *et al.* (2007) Single base errors in PCR products from avian museum specimens and their effect on estimates of historical genetic diversity. *Conserv. Genet.* 8, 879–884
54 Stuart, B.L. *et al.* (2006) Extraction of nuclear DNA from bone of skeletonized and fluid-preserved museum specimens. *Syst. Biodivers.* 4, 133–136
55 Williams, C. *et al.* (1999) A high frequency of sequence alterations is due to formalin fixation of archival specimens. *Am. J. Pathol.* 155, 1467–1471
56 Morin, P.A. *et al.* (2001) Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Mol. Ecol.* 10, 1835–1844
57 Gilbert, M.T.P. *et al.* (2005) Assessing ancient DNA studies. *Trends Ecol. Evol.* 20, 541–544
58 Morin, P.A. *et al.* (2004) SNPs in ecology, evolution and conservation. *Trends Ecol. Evol.* 19, 208–216
59 Morin, P.A. and Mc Carthy, M. (2007) Highly accurate SNP genotyping from historical and low-quality samples. *Mol. Ecol. Notes* 7, 937–946 (http://www.blackwell-synergy.com/loi/men)
60 Margulies, M. *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380
61 Poinar, H.N. *et al.* (2006) Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. *Science* 311, 392–394
62 Krause, J. *et al.* (2006) Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. *Nature* 439, 724–727
63 Panelli, S. *et al.* (2006) Towards the analysis of the genomes of single cells: further characterisation of the multiple displacement amplification. *Gene* 372, 1–7
64 Wang, G. *et al.* (2004) DNA amplification method tolerant to sample degradation. *Genome Res.* 14, 2357–2366
65 Schwartz, M.K. *et al.* (2007) Genetic monitoring as a promising tool for conservation and management. *Trends Ecol. Evol.* 22, 25–33
66 Manel, S. *et al.* (2003) Landscape genetics: combining landscape ecology and population genetics. *Trends Ecol. Evol.* 18, 189–197
67 Smulders, M.J.M. *et al.* (2003) Complete loss of MHC genetic diversity in the common hamster (*Cricetus cricetus*) population in The Netherlands. Consequences for conservation strategies. *Conserv. Genet.* 4, 441–451
68 Coughlan, J. *et al.* (2006) Temporal variation in an immune response gene (MHC I) in anadromous *Salmo trutta* in an Irish river before and during aquaculture activities. *ICES J. Mar. Sci.* 63, 1248–1255
69 Jaenicke-Despres, V. *et al.* (2003) Early allelic selection in maize as revealed by ancient DNA. *Science* 302, 1206–1208
70 Nielsen, R. (2005) Molecular signatures of natural selection. *Annu. Rev. Genet.* 39, 197–218
71 Denniston, C. and Crow, J.F. (1990) Alternative fitness models with the same allele frequency dynamics. *Genetics* 125, 201–205
72 Cozzolino, S. *et al.* (2007) Genetic variation in time and space: the use of herbarium specimens to reconstruct patterns of genetic variation in the endangered orchid *Anacamptis palustris*. *Conserv. Genet.* 8, 629–639
73 Harper, G.L. *et al.* (2006) Analysis of museum specimens suggests extreme genetic drift in the adonis blue butterfly (*Polyommatus bellargus*). *Biol. J. Linnean Soc.* 88, 447–452
74 Nielsen, E.E. *et al.* (1999) Analysis of DNA from old scale samples: technical aspects, applications and perspectives for conservation. *Hereditas* 130, 265–276
75 Thomas, W.K. *et al.* (1990) Spatial and temporal continuity of kangaroo rat populations shown by sequencing mitochondrial-DNA from museum specimens. *J. Mol. Evol.* 31, 101–112

76 Taylor, A.C. *et al.* (1994) Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Mol. Ecol.* 3, 277–290
77 Flagstad, O. *et al.* (2003) Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. *Mol. Ecol.* 12, 869–880
78 Mundy, N.I. *et al.* (1997) Skin from feet of museum specimens as a non-destructive source of DNA for avian genotyping. *Auk* 114, 126–129
79 Rohland, N. and Hofreiter, M. (2007) Comparison and optimization of ancient DNA extraction. *Biotechniques* 42, 343–352
80 Horváth, M.B. *et al.* (2005) An overlooked DNA source for non-invasive genetic analysis in birds. *J. Avian Biol.* 36, 84–88
81 Payne, R.B. and Sorenson, M.D. (2002) Museum collections as sources of genetic data. *Bonn. Zool. Beitr.* 51, 97–104
82 Gilbert, M.T.P. *et al.* (2007) DNA extraction from dry museum beetles without conferring external morphological damage. *PLoS One* DOI: 10.1371/journal.pone.0000272 (http://www.plosone.org)
83 Dean, M.D. and Ballard, J.W.O. (2001) Factors affecting mitochondrial DNA quality from museum preserved *Drosophila simulans*. *Entomol. Exp. Appl.* 98, 279–283
84 Drabkova, L. *et al.* (2002) Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of Juncaceae. *Plant Mol. Biol. Rep.* 20, 161–175
85 Walters, C. *et al.* (2006) The utility of aged seeds in DNA banks. *Seed Sci. Res.* 16, 169–178

ADDITIONAL PUBLICATION

THE NEED FOR A BETTER UNDERSTANDING OF INBREEDING EFFECTS ON POPULATION GROWTH

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COMMENTARY

The need for a better understanding of inbreeding effects on population growth

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Small populations are inherently at risk of stochastic fluctuations and inbreeding. These risks were recognized nearly 60 years ago (Dowdeswell, Fisher & Ford, 1949) but they did not receive widespread attention until the publication of two seminal text books on conservation biology (Soulé & Wilcox, 1980; Frankel & Soulé, 1981) in the early 1980s. Since then an increasing number of studies has shown that inbreeding depression is pervasive in natural populations (Keller & Waller, 2002), at times even among species that are known to inbreed regularly (Ross-Gillespie, O'Riain & Keller, in press).

Inbreeding depression in individual fitness components is, however, of limited importance to conservation biology unless these reductions in individual fitness translate into reduced population growth rates. That inbreeding can potentially reduce population growth rates and increase extinction risks was demonstrated by influential studies of experimental plant populations (Newman & Pilson, 1997) and a butterfly metapopulation (Saccheri *et al*., 1998) and, more recently, by studies that demonstrate how experimentally restored immigration rapidly reverses negative population growth rates of inbred populations (Hogg *et al*., 2006). By demonstrating a link between population size (as a proxy of genetic variation) and population growth rates Reed, Nicholas & Stratton's (2007) study adds to the growing evidence that inbred populations may experience reduced population growth rates.

Small and inbred populations do not always, however, experience reduced population growth rates (Broders *et al*., 1999). Why would inbreeding depression in individual fitness not always translate into a reduced population growth rate? The strongest argument is that of soft selection (Saccheri & Hanski, 2006). Under many circumstances, the probability of survival of an individual may depend on the presence or absence of other individuals. For example, in a territorial species a proportion of juveniles might die (or emigrate) simply because all territories are occupied by stronger competitors. Had fewer strong competitors existed, all individuals might have acquired territories. That is, the selection is both density- and frequency-dependent. Borrowing terminology from the international monetary exchange, selection that is both density and frequency-dependent was coined 'soft selection' by Wallace (1970, 1975). In the context of inbreeding depression, soft selection implies that breeding territories are filled by the least inbred individuals. In a large population these might be outbred individuals but in a very small population these individuals might be appreciably inbred. In the absence of any fitter competitors, these inbred individuals may produce enough offspring so that inbreeding depression in individual fitness has negligible effects on population size (Wallace, 1970, 1975). Thus, if soft selection predominates in natural populations, inbreeding may reduce population growth rates less than individual fitness.

Hard selection, on the other hand, describes selection that is neither density- nor frequency-dependent (Wallace, 1970, 1975). Unconditionally lethal genes are one example of hard selection. If unconditionally lethal genes are a major source of inbreeding depression, then hard selection predominates and inbreeding depression in individual fitness would reduce population size. Despite their importance, we have few estimates of the numbers of unconditionally lethal genes in natural populations. Two estimates from wild fish species suggest, however, that this number is relatively small (1–2 per individual) and comparable to estimates from laboratory studies on *Drosophila* and *Xenopus laevis* (McCune *et al*., 2002). Thus, while some unconditionally lethal genes will undoubtedly be involved, purging is likely to remove such lethals from many populations and a substantial part of inbreeding depression in individual fitness traits is expected to be caused by genes of minor effect (Willis, 1999). Thus, the evidence we have on the genetic architecture of inbreeding depression to date suggests that hard selection is not an inevitable consequence of inbreeding. Note also, that in the context of structured populations or metapopulations, soft selection represents population regulation at the level of the local subpopulation while hard selection

represents regulation at the metapopulation level (Whitlock, 2002; Saccheri & Hanski, 2006).

These considerations allow us to predict under which circumstances we would expect inbreeding depression in individual fitness to translate more strongly into reduced population growth rates. For example, populations that exhibit low levels of density dependence and those that form part of a metapopulation are expected to show stronger effects of inbreeding on population growth rates, as would populations with higher genomic numbers of unconditional lethals. The study of Saccheri *et al*. (1998) fits this expectation, since the Glanville fritillary butterflies on Åland form part of a metapopulation that is regulated at the metapopulation level. The two spider species studied by Reed *et al*. (2007), on the other hand, do not seem to fit the expectations: there was evidence for density dependence in survival. However, the populations of *Rabidosa rabida* exchange up to 1.5 migrants per generation, suggesting that they might form part of a metapopulation. If this conjecture should be true and if these populations are regulated at the metapopulation level, these aspects of the spiders' biology may explain the evidence for reduced population growth rates among the smaller populations. Estimates of the magnitude of inbreeding depression and of the details of the processes that regulate these spider populations are required to explain the observed effects in detail.

Reed *et al*.'s (2007) study highlights another important aspect: that inbreeding and environmental effects may interact in their effects on population dynamics. That is, population size (as a proxy of genetic variation) affected population growth rates the most in years when prey availability was decreasing. Such synergistic effects of inbreeding and environmental stressors on extinction probabilities are well known from laboratory experiments with *Drosophila* (Bijlsma, Bundgaard & Boerema, 2000) and from theoretical models (Tanaka, 1998). The likelihood of such synergistic interactions may well turn out to depend on the soft–hard selection continuum, too. The two studies mentioned above suggest this tantalizing conclusion: The models imposed hard selection in that inbreeding affected population growth rates directly (Tanaka, 1998). And in *Drosophila*, some lethal genes are known to be expressed only under certain environmental conditions (Vermeulen & Bijlsma, 2004), suggesting that environmental stressors can change the genetic architecture of inbreeding depression to one favouring hard selection.

Much of this is still conjecture because we lack data on many of the relevant variables, particularly in natural populations. Defining the conditions under which population growth rates are depressed by inbreeding will remain one of the major challenges for conservation genetics today.

References

Bijlsma, R., Bundgaard, J. & Boerema, A.C. (2000). Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila*. *J. Evol. Biol.* **13**, 502–514.

Broders, H.G., Mahoney, S.P., Montevecchi, W.A. & Davidson, W.S. (1999). Population genetic structure and the effect of founder events on the genetic variability of moose, *Alces alces*, in Canada. *Mol. Ecol.* **8**, 1309–1315.

Dowdeswell, W.H., Fisher, R.A. & Ford, E.B. (1949). The quantitative study of populations in the *Lepidoptera*.2. *Maniola jurtina* L. *Heredity* **3**, 67–84.

Frankel, O.H. & Soulé, M.E. (1981). *Conservation and Evolution*. Cambridge: Cambridge University Press.

Hogg, J.T., Forbes, S.H., Steele, B.M. & Luikart, G. (2006). Genetic rescue of an insular population of large mammals. *Proc. Roy. Soc. B – Biol. Sci.* **273**, 1491–1499.

Keller, L.F. & Waller, D.M. (2002). Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**, 230–241.

McCune, A.R., Fuller, R.C., Aquilina, A.A., Dawley, R.M., Fadool, J.M., Houle, D., Travis, J. & Kondrashov, A.S. (2002). A low genomic number of recessive lethals in natural populations of bluefin killifish and zebrafish. *Science* **296**, 2398–2401.

Newman, D. & Pilson, D. (1997). Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. *Evolution* **51**, 354–362.

Reed, D.H., Nicholas, A.C. & Stratton, G.E. (2007). Genetic quality of individuals impacts population dynamics. *Anim. Conserv.* **10**, 275–283.

Ross-Gillespie, A., O'Riain, M.J. & Keller, L.F. (in press) Viral epizootic reveals inbreeding depression in a habitually inbreeding mammal. *Evolution*.

Saccheri, I. & Hanski, I. (2006). Natural selection and population dynamics. *Trends Ecol. Evol.* **21**, 341–347.

Saccheri, I., Kuussaari, M., Kankare, M., Vikman, P., Fortelius, W. & Hanski, I. (1998). Inbreeding and extinction in a butterfly metapopulation. *Nature* **392**, 491–494.

Soulé, M.E. & Wilcox, B. (1980). *Conservation biology: An evolutionary–ecological perspective*. Sunderland: Sinauer Associates.

Tanaka, Y. (1998). Theoretical aspects of extinction by inbreeding depression. *Res. Pop. Ecol.* **40**, 279–286.

Vermeulen, C.J. & Bijlsma, R. (2004). Characterization of conditionally expressed mutants affecting age-specific *Drosophila melanogaster*: lethal conditions and temperature-sensitive periods. *Genetics* **167**, 1241–1248.

Wallace, B. (1970). *Genetic load: its biological and conceptual aspects*. Englewood Cliffs, NJ: Prentice Hall.

Wallace, B. (1975). Hard and soft selection revisited. *Evolution* **29**, 465–473.

Whitlock, M.C. (2002). Selection, load and inbreeding depression in a large metapopulation. *Genetics* **160**, 1191–1202.

Willis, J.H. (1999). The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. *Evolution* **53**, 1678–1691.

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